

# Fundamentals, Optimization and Practical Aspects of (U)HPLC

Definitions and basic concepts of Ultra High Performance Liquid Chromatography

# 2004: HPLC evolved to UHPLC

Two key ingredients for this evolution:

- Introduction of new, ultra-high pressure stable, sub-2-µm diameter particles in 2004 followed by the introduction of superficially porous, low diameter particles in 2006
- Next generation HPLC instrumentation capable to deliver solvents at ultra-high pressure and able to conserve the ultra high efficiency separation of columns packed with these new particles

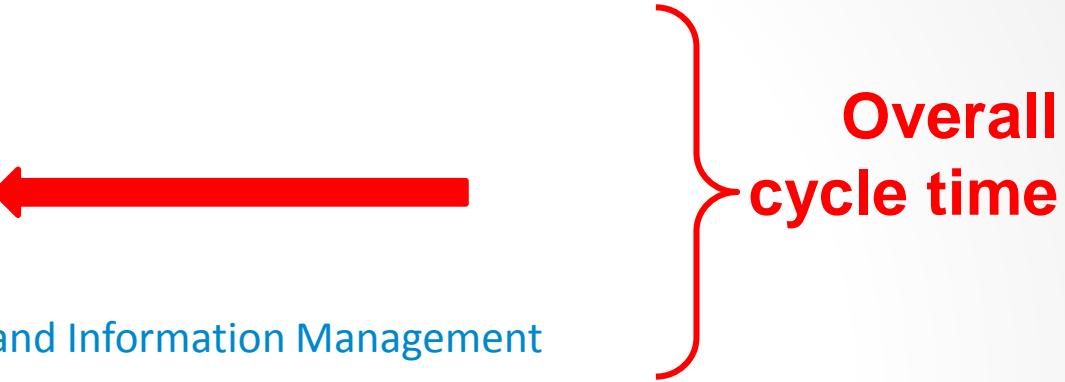
# Key ingredients of UHPLC:

- New Column Technologies:
  - Totally porous (**TP**) sub-2-micrometer particles
  - Superficially porous (**SP**) particles
  - (Monolithic columns)
- Next generation HPLC instrumentation:
  - Delivering mobile phases at ultra high pressure (now up to 1500 bar)
  - Systems optimized to conserve peak fidelity before, during and after separation
- Does this mean that UHPLC differs from HPLC?

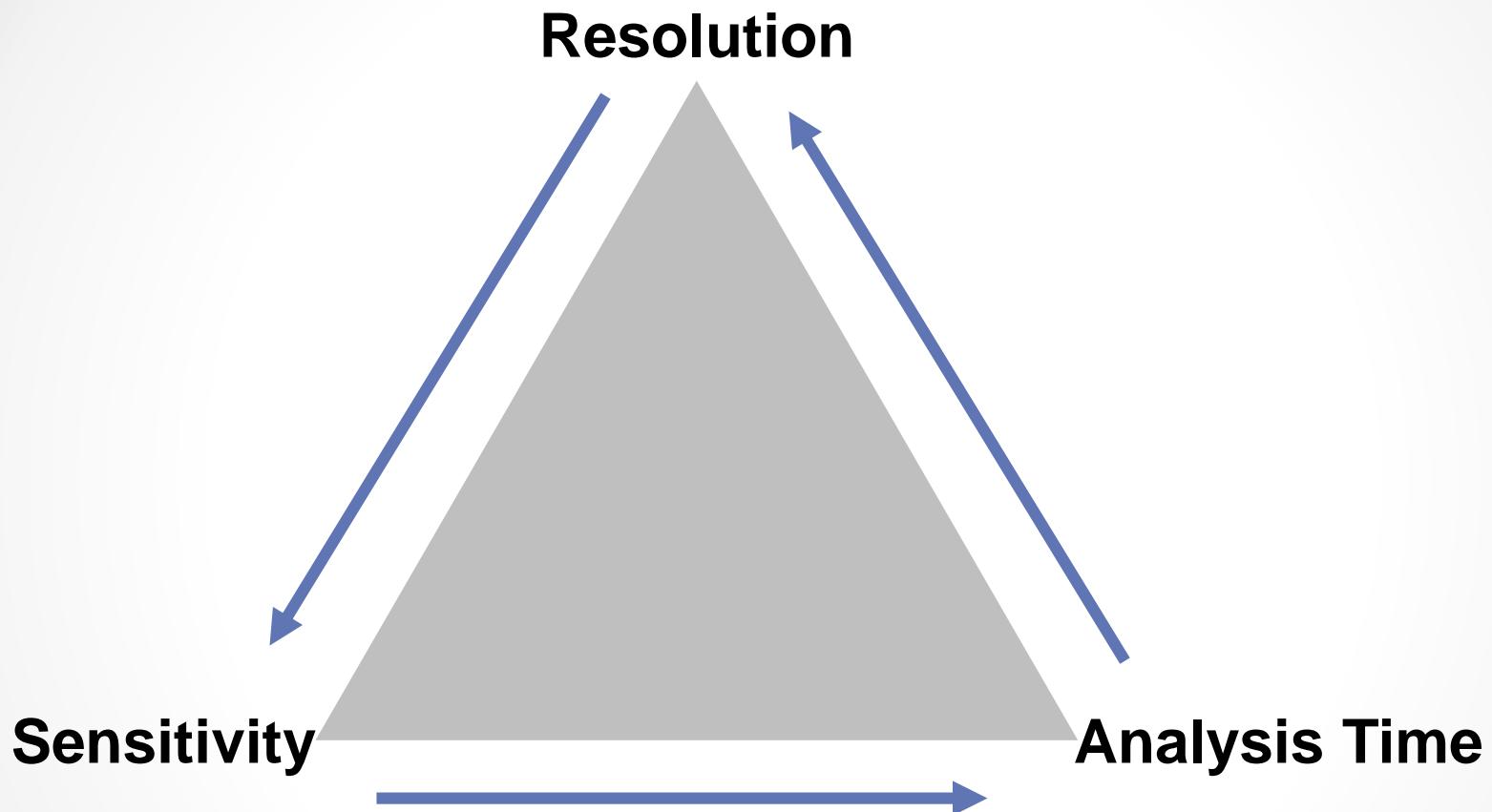
→ no!
- Basic theory of HPLC applies as well to UHPLC

Mind this nomenclature!

# Considerations Before Optimizing an (U)HPLC Separation

- Should Comprise all Operations of an HPLC Analysis
    - Sample Preparation
    - Sample Handling and Injection
    - Chromatographic Separation
    - Detection
    - Quantitation, Data Evaluation and Information Management
  - Should Meet User Requirements
    - Robustness; towards slight change in conditions
    - Reliability, stability and longevity
    - Reproducibility and accuracy
    - Affordable
    - Ease of use; match skill of the operators
  - Must Meet Internal and External Compliance and Regulatory Requirements (OQPV, Validation, ISO, GLP, etc.)
- 
- Overall cycle time**

# The Chromatography Optimization “Trilemma”

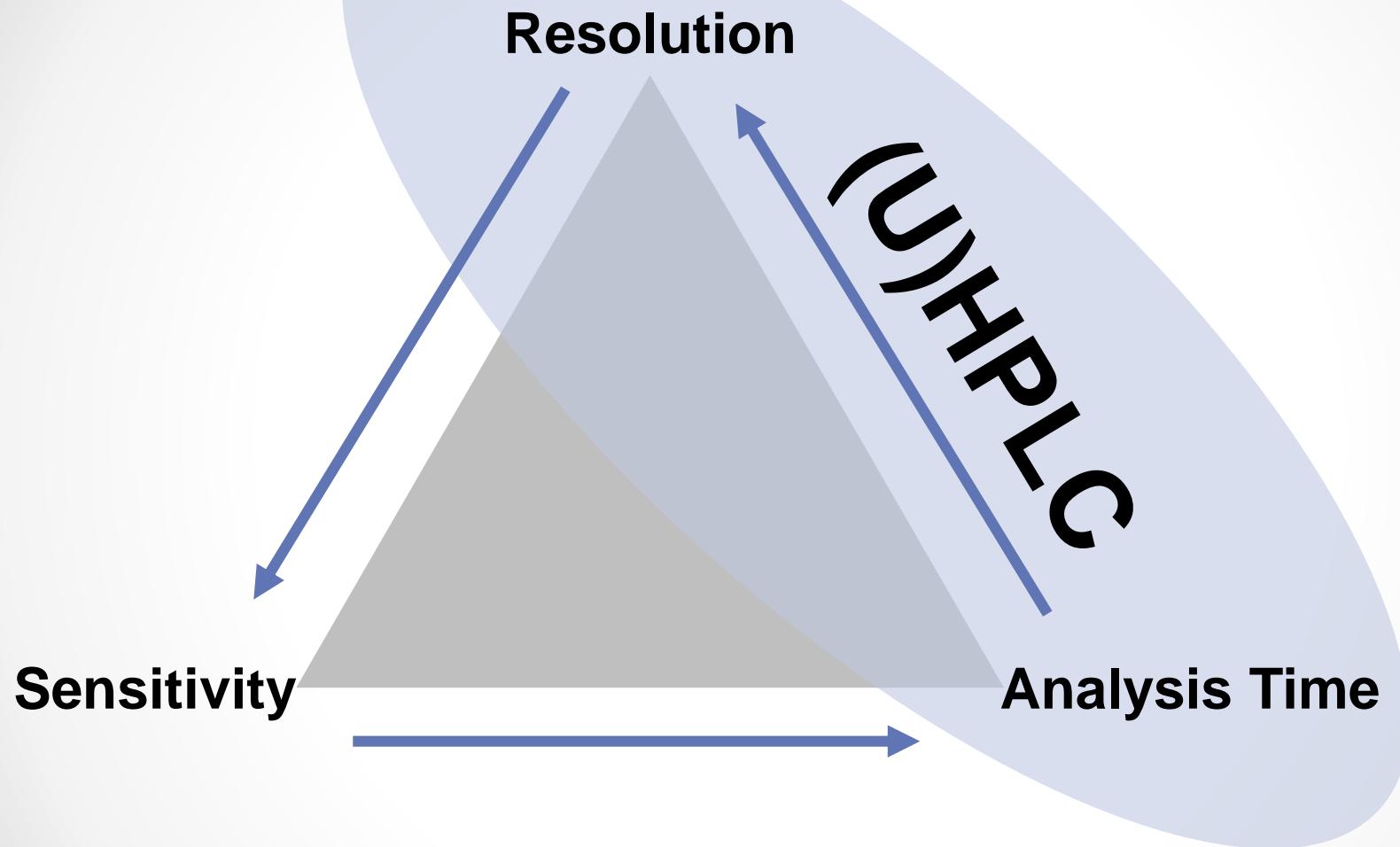


Quoted from Prof. Georges Guiochon\*

*“the primary objective of an analysis by HPLC separation is to identify a maximum number of analytes (**resolution**) in the minimum amount of time ( $t_0$ ) and to derive an accurate quantitative estimate of their concentrations (**sensitivity**)."*

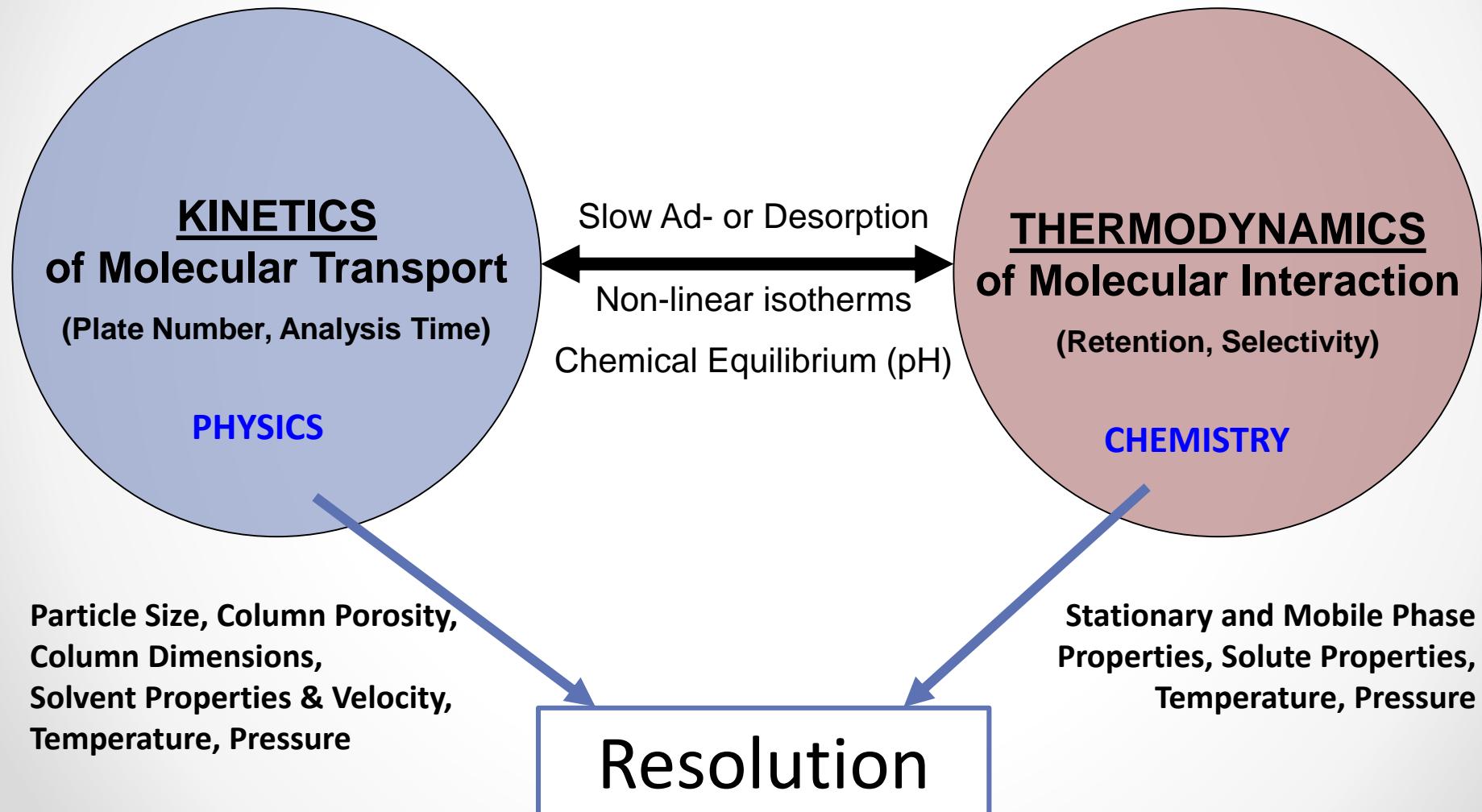
\*F. Gritti and G. Guiochon, J. Chromatography A, 1228 (2012) 2–19; Prof. Guiochon passed away, October 21, 2014

# The Chromatography Optimization “Trilemma”

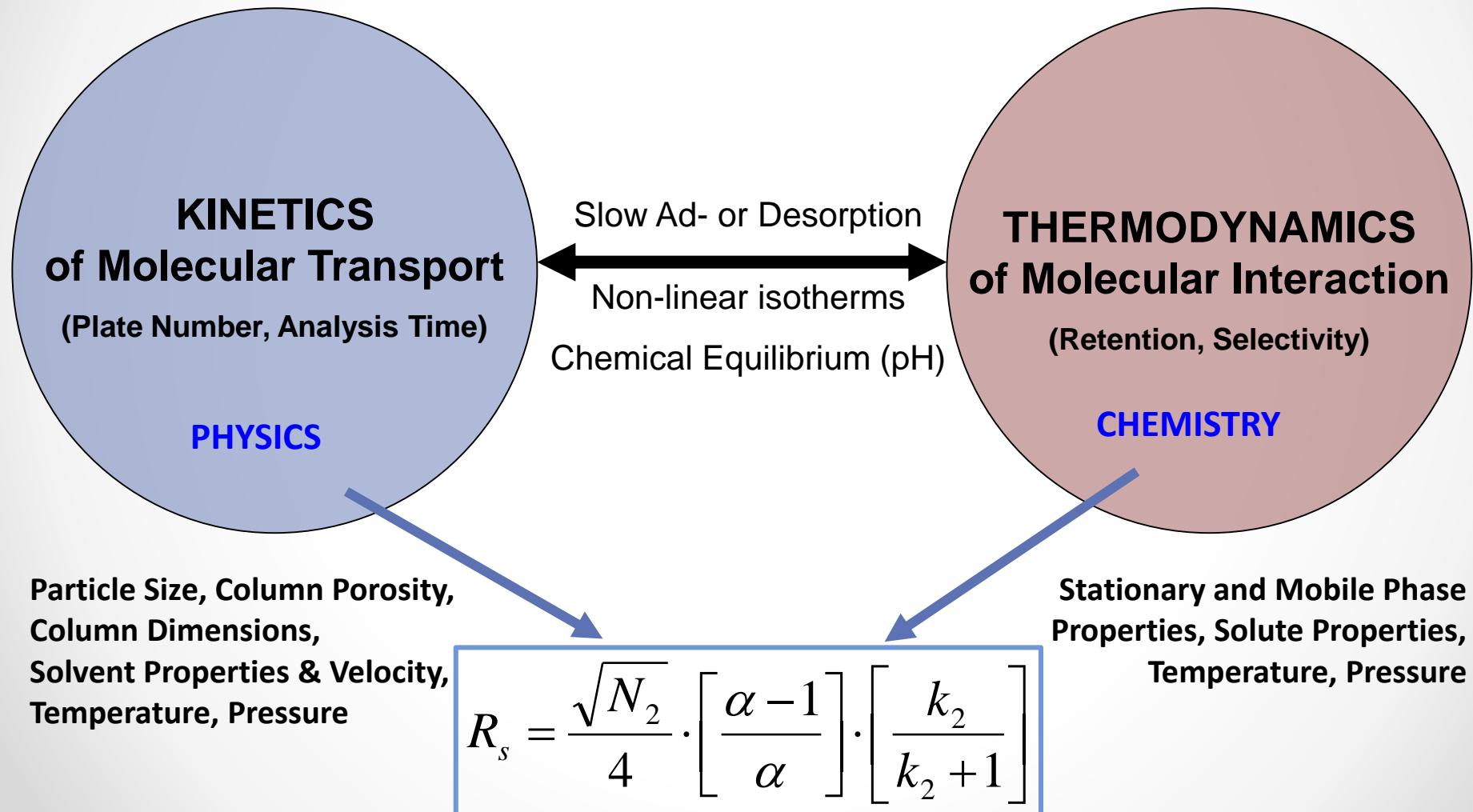


**Primary Goal of (U)HPLC:**  
Obtain the best possible resolution in the shortest possible time

# Mechanisms of Separation in HPLC



# Mechanism of Separation in HPLC



# Improve Resolution by Selectivity

- Selectivity ( $\alpha$ ) helps best but:

$$R_s = \frac{\sqrt{N_2}}{4} \cdot \left[ \frac{\alpha - 1}{\alpha} \right] \cdot \left[ \frac{k_2}{k_2 + 1} \right]$$

- Is driven by difference in molecular properties (polarity, charge, hydrophobicity, size etc.)

- “Know your molecules”!!

- Is difficult to predict

- Experience helps (ask your colleague, check the literature!  
(<http://scholar.google.com>, <http://academic.research.microsoft.com/>)

- Establish retention model for all molecules one is interested in

- Use of optimization software for the separation of multi-component mixtures (ChromSword, DryLab)

- May become a laborious procedure especially with multicomponent samples
  - Impossible to do with very complex samples >50 components (e.g. tryptic digests, biomedical or environmental samples)

# Improve Resolution → Increase Plate Number

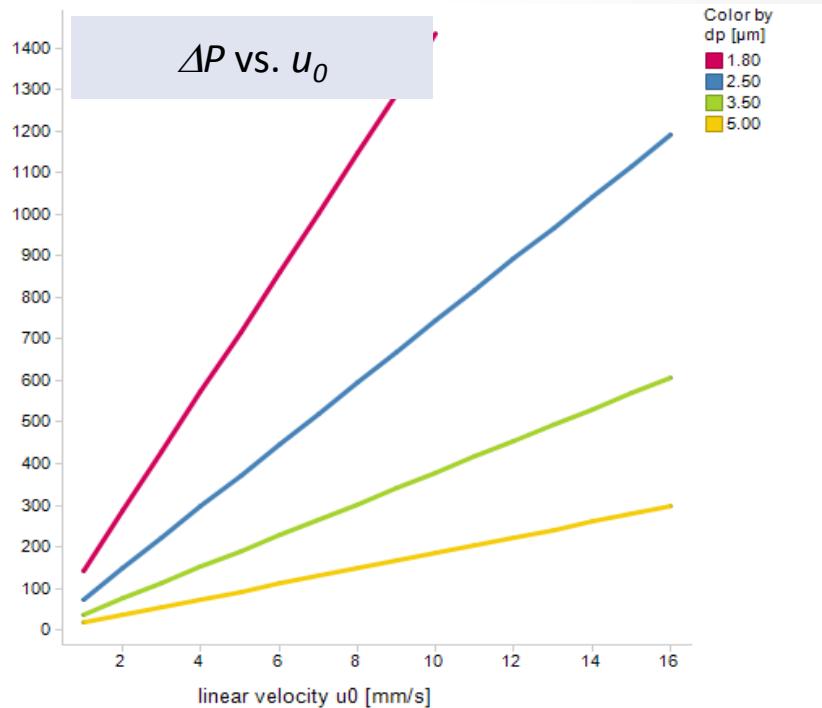
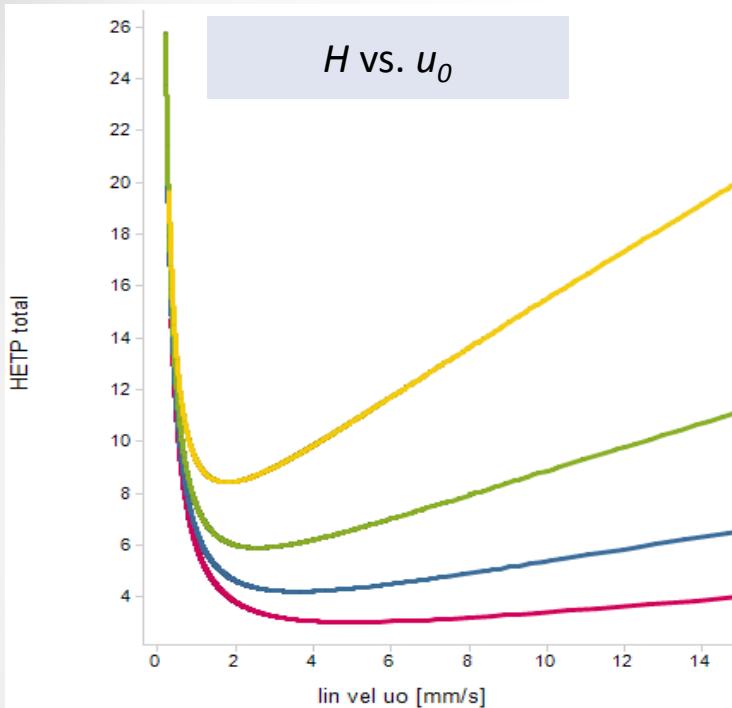
$$R_s = \frac{\sqrt{N_2}}{4} \cdot \left[ \frac{\alpha - 1}{\alpha} \right] \cdot \left[ \frac{k_2}{k_2 + 1} \right]$$



$$N = \frac{L}{H}$$

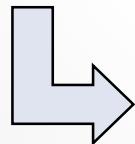
Action	Constraint
Increase length of the column	Longer analysis time Higher column pressure
Decrease H(ETP) by using smaller particles	Higher column pressure

# HETP and Pressure Drop vs. Solvent Velocity

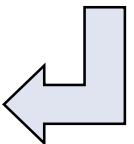


$$t_0 = \frac{L}{u_0}$$

$$N = L/H$$



Classical Dilemma



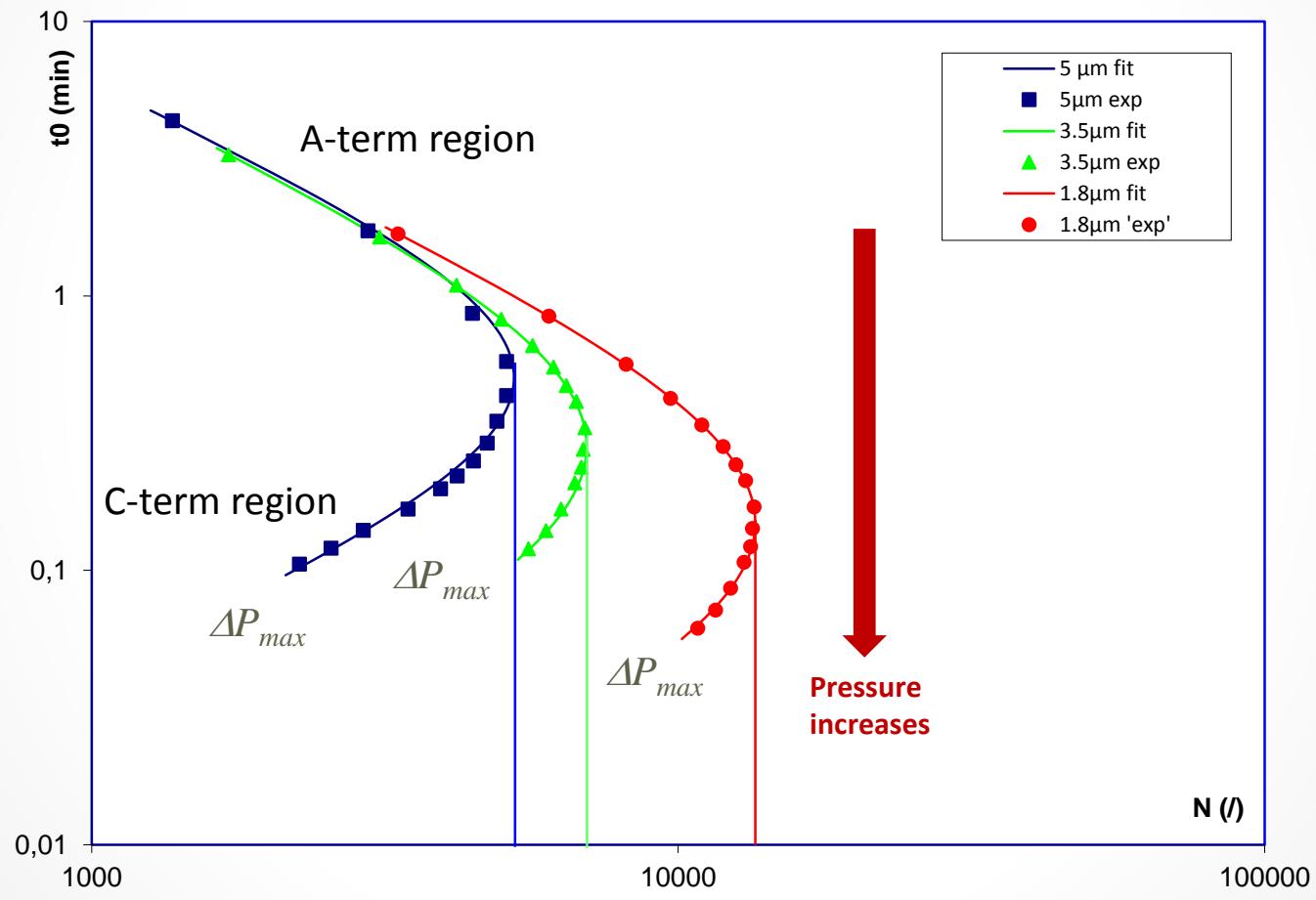
$$\Delta P_{\max} = u_0 \cdot \eta \cdot L \cdot \frac{\Phi_0}{d_p^2}$$

Column length = 100 mm, viscosity = 1 cP

# UHPLC - Essentials

## van Deemter Plot reordered\*

$L$  (50 mm) and  $d_p$  fixed, select optimal  $u_o$



\*Slide courtesy of Prof. Ken Broeckhoven, Free University of Brussels

# Classical Dilemma of HPLC

How to achieve a particular plate number  $N_{\text{required}}$  ( $N_r$ ), say 20,000 plates, in the shortest time possible at a given maximal column pressure available?

According to J.H. Knox and M. Saleem,  
J. Chromatogr. Sci., 7 (1969), p. 614

# How to Obtain the Required Plate Number in the Shortest Time?

<b>200 bar</b>			
$N_r$	$t_0$	$d_p$	$L$
1000	0.2 s	0.5 $\mu\text{m}$	1.1 mm
10000	20 s	1.7 $\mu\text{m}$	35 mm
100000	2000 s	5 $\mu\text{m}$	1100 mm
1000000	2.3 days	17 $\mu\text{m}$	35 m
<b>400 bar</b>			
1000	0.1 s	0.39 $\mu\text{m}$	0.78 mm
10000	10 s	1.22 $\mu\text{m}$	24.4 mm
100000	1000 s	3.87 $\mu\text{m}$	774 mm
1000000	1.16 days	12.25 $\mu\text{m}$	24.5 m
<b>1000 bar</b>			
1000	0.04 s	0.25 $\mu\text{m}$	0.5 mm
10000	4 s	0.77 $\mu\text{m}$	15.5 mm
100000	400 s	2.45 $\mu\text{m}$	490 mm
1000000	0.46 days	7.75 $\mu\text{m}$	15.5 m

Unretained retention, *optimal particle size* and column length at pressure limited situations in HPLC

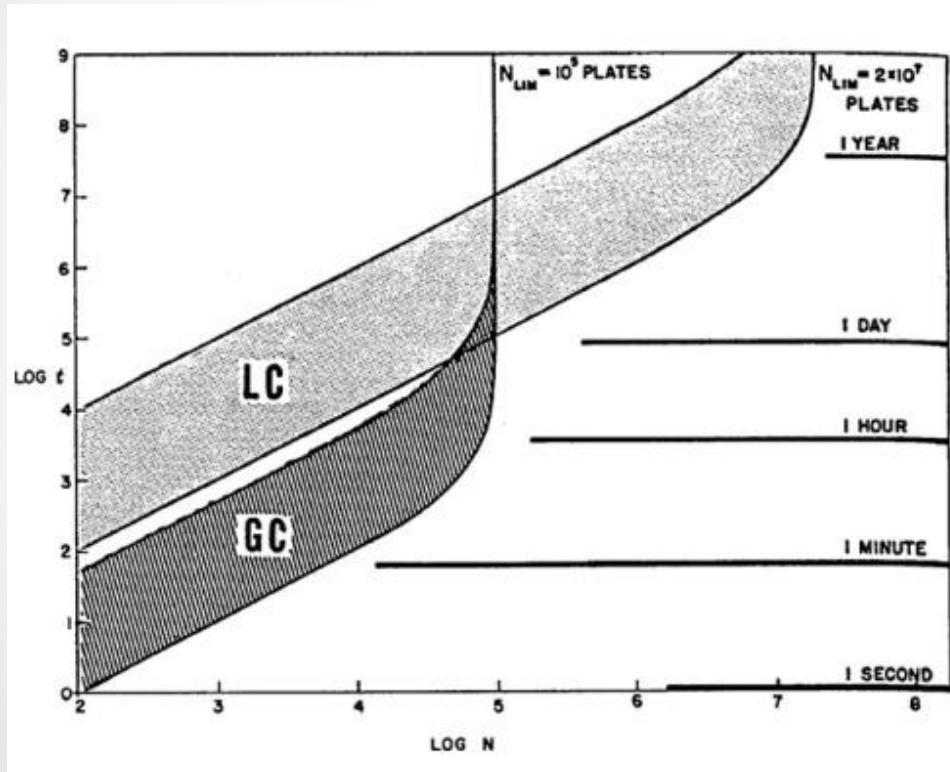
# In Order to Get Separation.....

- Keep in mind that selectivity works best in obtaining resolution for samples with few compounds, but may require a laborious optimization procedure.
- The objective in (U)HPLC is to obtain the best resolution in the shortest possible time.
- Very high efficiencies ( $N > 50,000$ ) require bigger particles and longer columns at the cost of time
- Medium efficiencies ( $N < 50,000$ ) satisfying in 99% of all cases, are obtained in short time with very small particles and short column lengths at the cost of pressure

# Kinetic Plot Theory

## “Kinetic Performance Limit” Plot Approach

Originally proposed by Giddings\*



\*J.C. Giddings, Anal. Chem. 37, 60 (1965)

# Kinetic Plot Theory

## “Kinetic Performance Limit” Plot Approach

Equations required:

$$\left\{ \begin{array}{l} L = N.H \\ t_0 = \frac{L}{u_0} = \frac{N.H}{u_0} \\ u_0 = B_0 \cdot \frac{\Delta P}{\eta \cdot L} = B_0 \cdot \frac{\Delta P}{\eta \cdot H \cdot N} \end{array} \right. \quad \begin{matrix} \longrightarrow \\ \longrightarrow \end{matrix} \quad \begin{array}{l} t_0 = \left\{ \frac{\Delta P}{\eta} \right\} \cdot \left\{ \frac{B_0}{u_0^2} \right\} \\ N = \left\{ \frac{\Delta P}{\eta} \right\} \cdot \left\{ \frac{B_0}{u_0 H} \right\} \end{array}$$

\*J.C. Giddings, Anal. Chem. **37**, 60 (1965)

\*\*G. Desmet et al., LCGC Europe, **18** (7), 403-409 (2005) and G. Desmet et al., J. Chrom. A, **1228**, 20 (2012);

# Kinetic Plot Theory

## “Kinetic Performance Limit” Plot Approach\*

Example:

Maximum pressure 400 bar  
Column Zorbax 3.5µm 10cm

Experimental data			
$F$ (ml/min)	$\Delta P$ (bar)	$u_0$ (mm/s)	$H$ (µm)
3,5	228,1	6,95	8,72
3	196,5	5,97	8,40
2,5	164,6	4,98	7,92
2	132,8	4,00	7,51
1,75	117,0	3,51	7,31
1,5	101,2	3,01	7,26
1,25	85,2	2,52	7,21
1	69,1	2,02	7,46
0,875	61,1	1,77	7,75
0,75	53,0	1,51	8,15
0,625	45,0	1,26	8,86
0,5	36,7	1,01	10,02
0,375	28,4	0,76	11,93
0,25	19,6	0,51	16,15
0,125	10,4	0,25	29,21

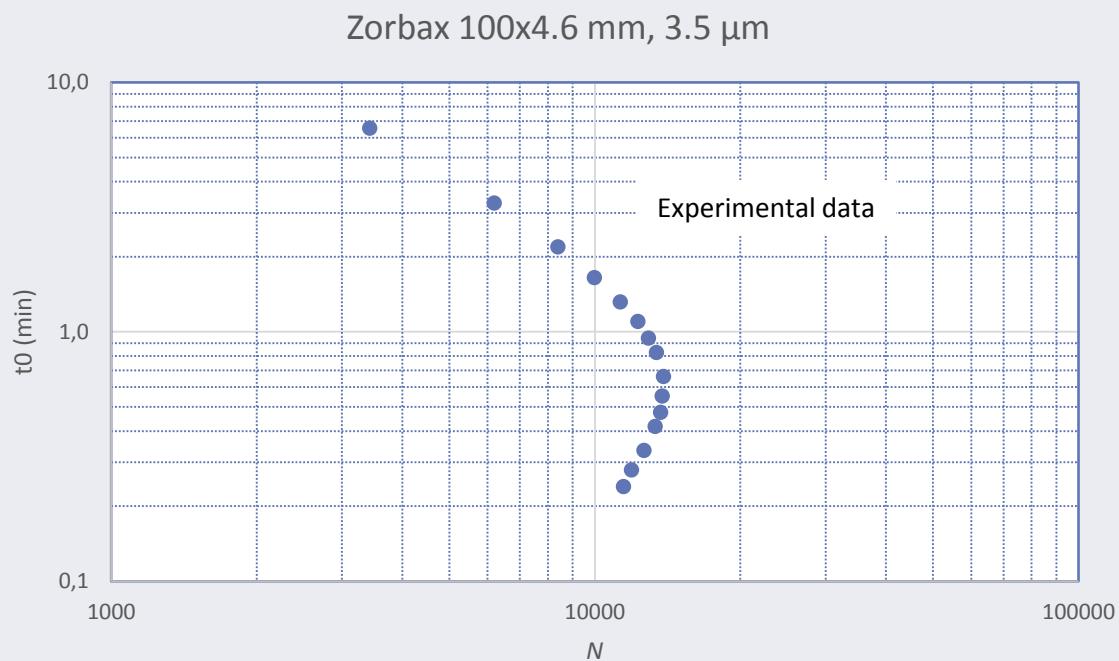
Data courtesy of Profs. Gert Desmet & Ken Broeckhoven, Free University Brussels

# Kinetic Plot Theory

## “Kinetic Performance Limit” Plot Approach

### Experimental data

Experimental data					
$F$ (ml/min)	$\Delta P$ (bar)	$u_0$ (mm/s)	$H$ ( $\mu\text{m}$ )	$t_0$ (min)	$N$
3,5	228,1	6,95	8,72	0,240	11466
3	196,5	5,97	8,40	0,279	11911
2,5	164,6	4,98	7,92	0,335	12633
2	132,8	4,00	7,51	0,417	13323
1,75	117,0	3,51	7,31	0,475	13675
1,5	101,2	3,01	7,26	0,553	13779
1,25	85,2	2,52	7,21	0,662	13860
1	69,1	2,02	7,46	0,826	13408
0,875	61,1	1,77	7,75		
0,75	53,0	1,51	8,15		
0,625	45,0	1,26	8,86		
0,5	36,7	1,01	10,02		
0,375	28,4	0,76	11,93		
0,25	19,6	0,51	16,15		
0,125	10,4	0,25	29,21		



Data courtesy of Profs. Gert Desmet & Ken Broeckhoven, Free University Brussels

# Kinetic Plot Theory

## “Kinetic Performance Limit” Plot Approach\*

Extrapolate the experimental data  $N$ ,  $t_0$  (or  $H, u_0$ ) obtained by measuring a “van Deemter” plot, to the kinetic performance limit (KPL) obtained at  $\Delta P_{max}$

Elongation factor:  $\lambda = \frac{\Delta P_{max}}{\Delta P_{exp}}$

$$N_{KPL} = \lambda \cdot N_{exp}$$

$$t_{0,KPL} = \lambda \cdot t_{0,exp}$$

$$L_{KPL} = \lambda \cdot L_{exp}$$

$$t_{R,KPL} = \lambda \cdot t_{R,exp}$$

\* K. Broeckhoven, et al., J. Chromatogr. A, 1217 (2010) 2787-2795.

# Kinetic Plot Theory

## “Kinetic Performance Limit” Plot Approach

$$\Delta P_{max} = 400 \text{ bar}$$

Experimental data						Kinetic plot data			
F (ml/min)	$\Delta P$ (bar)	$u_0$ (mm/s)	H ( $\mu\text{m}$ )	$t_0$ (min)	N (/)	$\lambda$ (/)	$t_0$ KPL (min)	N KPL (min)	$t_R$ KPL (min)
3,5	228,1	6,95	8,72	0,240	11466	1,75	0,420	20109	2,98
3	196,5	5,97	8,40	0,279	11911	2,04	0,568	24247	4,03
2,5	164,6	4,98	7,92	0,335	12633	2,43	0,814	30699	5,78
2	132,8	4,00	7,51	0,417	13323	3,01	1,256	40118	8,92
1,75	117,0	3,51	7,31	0,475	13675	3,42	1,625	46745	11,54
1,5	101,2	3,01	7,26	0,553	13779	3,95	2,187	54464	15,53
1,25	85,2	2,52	7,21	0,662	13860	4,69	3,108	65047	22,07
1	69,1	2,02	7,46	0,826	13408	5,79	4,786	77654	33,98
0,875	61,1	1,77	7,75	0,944	12902	6,55	6,182	84490	43,89
0,75	53,0	1,51	8,15	1,101	12268	7,54	8,309	92560	59,00
0,625	45,0	1,26	8,86	1,319	11293	8,89	11,727	100382	83,26
0,5	36,7	1,01	10,02	1,647	9983	10,89	17,946	108754	127,42
0,375	28,4	0,76	11,93	2,192	8382	14,11	30,928	118265	219,59
0,25	19,6	0,51	16,15	3,284	6191	20,46	67,199	126663	477,11
0,125	10,4	0,25	29,21	6,553	3423	38,40	251,635	131456	1786,61

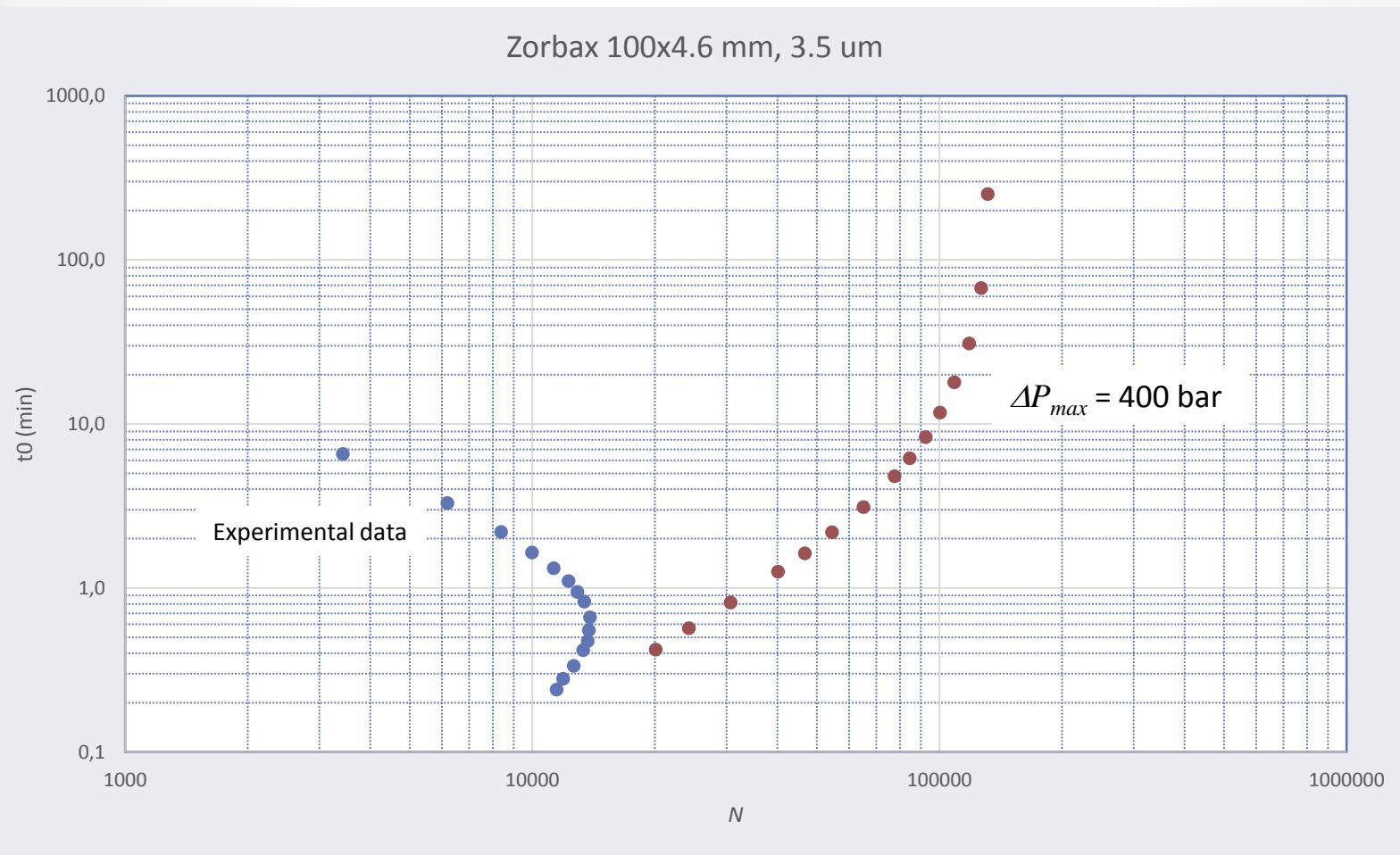


Elongation factor  $\lambda$

Data courtesy of Profs. Gert Desmet & Ken Broeckhoven, Free University Brussels

# Kinetic Plot Theory

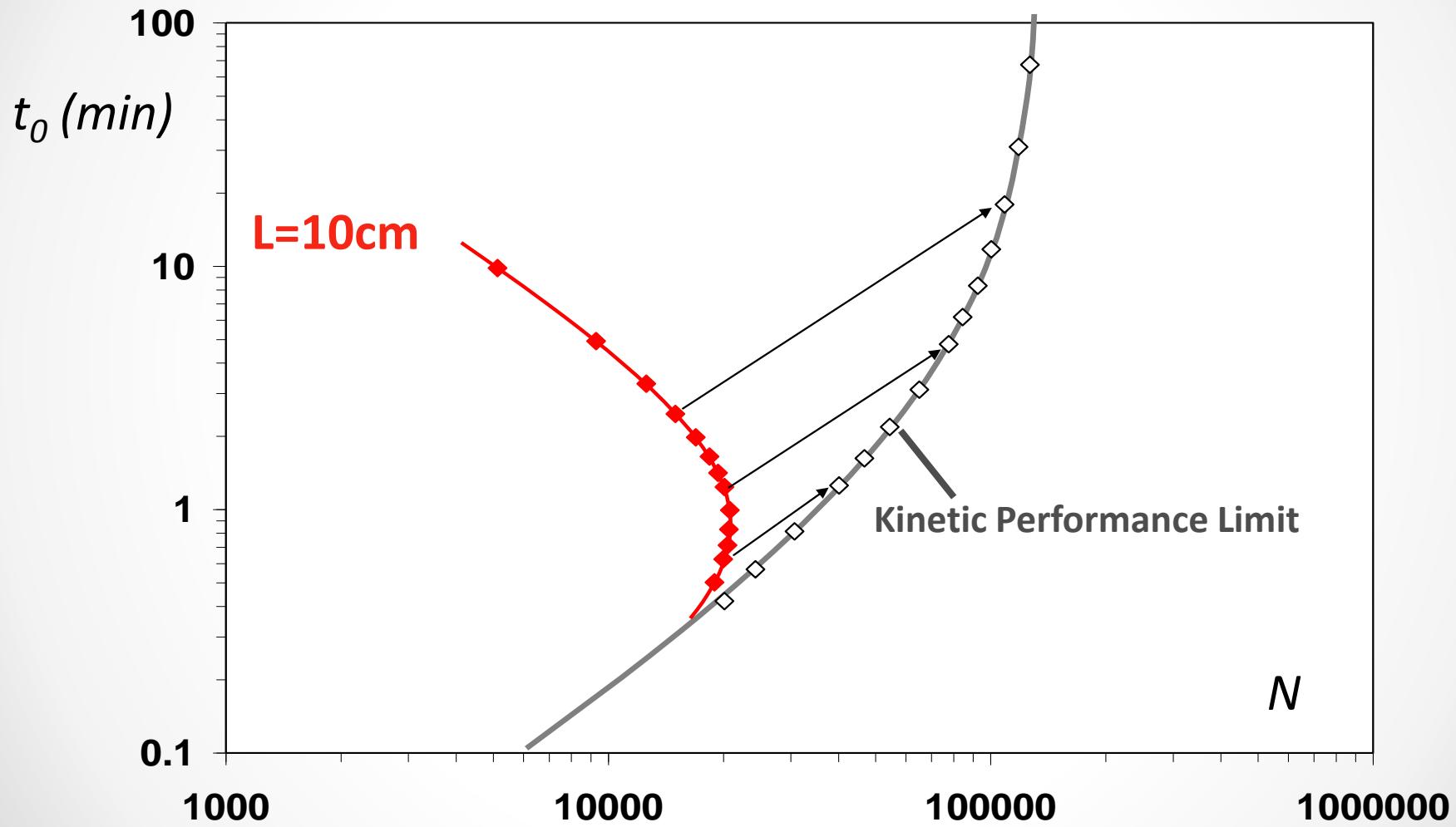
## “Kinetic Performance Limit” Plot Approach



Data courtesy of Profs. Gert Desmet & Ken Broeckhoven, Free University Brussels

# Kinetic Plot Theory

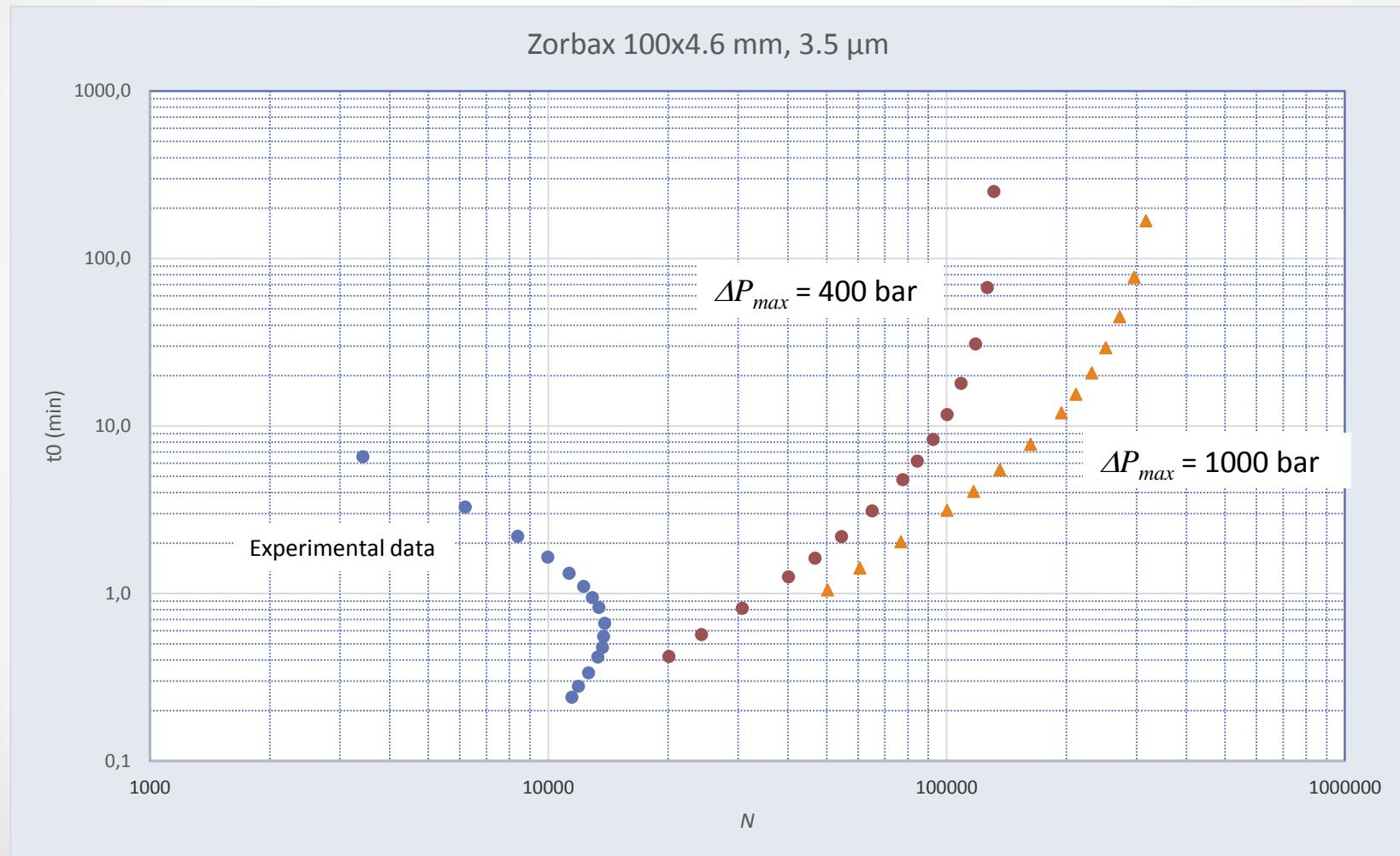
## “Kinetic Performance Limit” Plot Approach



Data courtesy of Profs. Gert Desmet & Ken Broeckhoven, Free University Brussels

# Kinetic Plot Theory

## “Kinetic Performance Limit” Plot Approach



# Role of Temperature in (U)HPLC

## Temperature increase

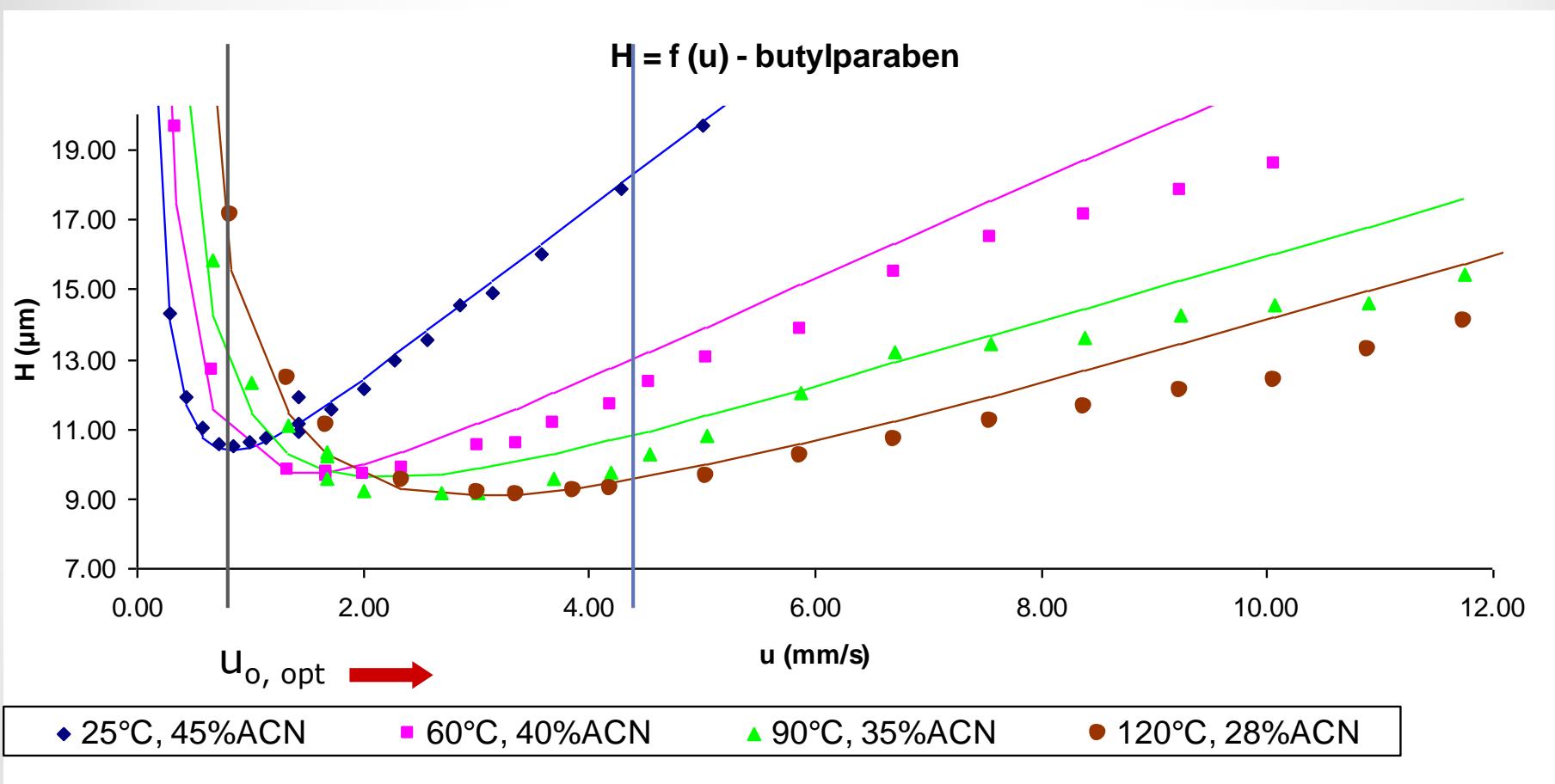
- Decrease in the mobile phase viscosity\*
  - Viscosity is reduced 1 to 2 % per °C increase
  - Lower back pressure
- Increase of solute diffusivity
  - Lower HETP-value
- Decrease in the mobile phase polarity
  - Increasing temperature 4 to 5 °C is comparable to increasing the methanol or acetonitrile concentration by 1% in a reversed phase system
  - Less organic solvent in the eluent (in RP separations)

$$D_{m,T} = D_{m,298} \frac{\eta_{298}}{\eta_T} \frac{T}{298}$$

\*J. Billen et al., J. Chrom. A, 1210, 30 (2008)

# Role of Temperature in (U)HPLC

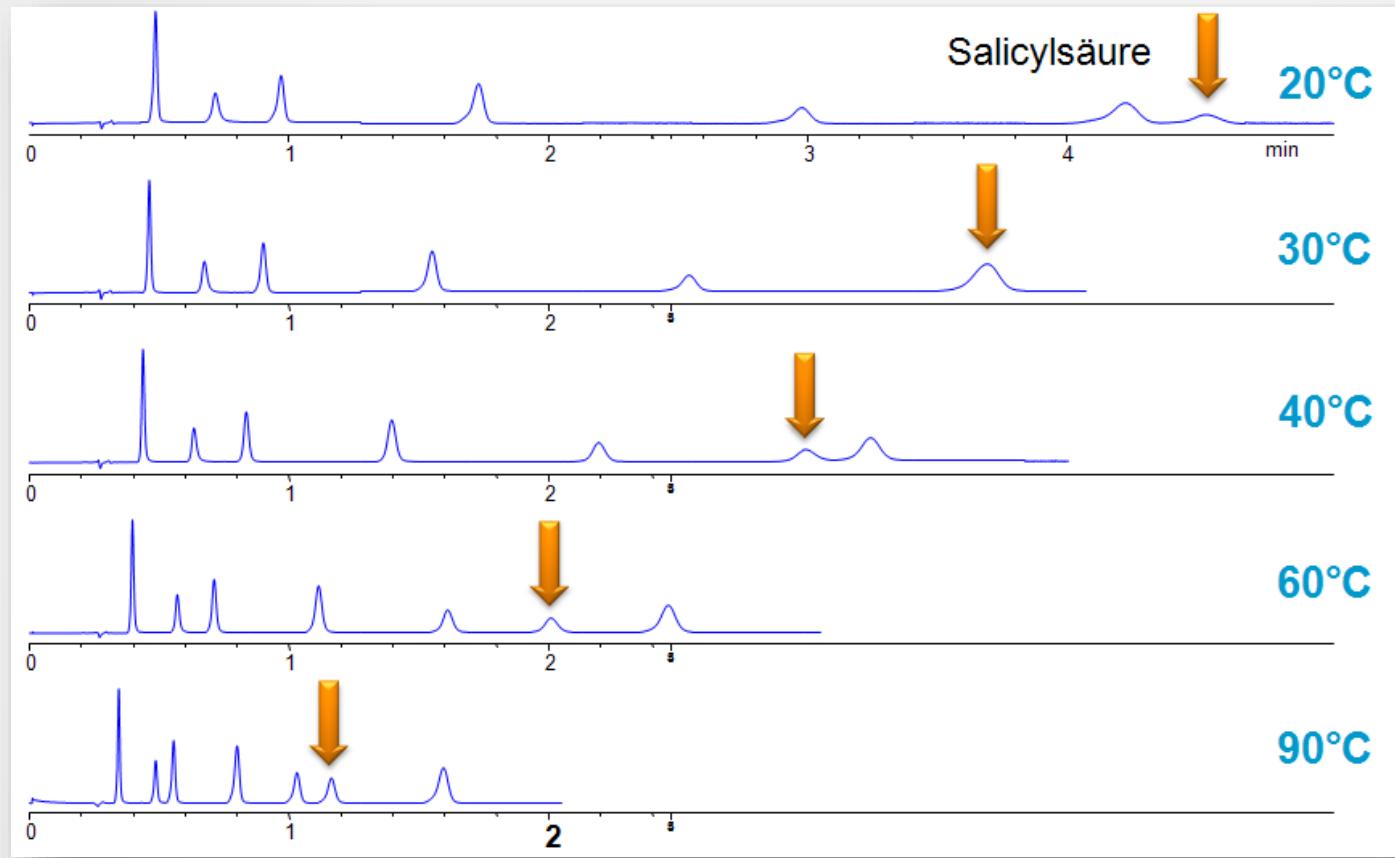
## Experimental Result\*



\*Results courtesy of Dr. Davy Guillarme, Univ. Geneva

# Role of Temperature in UHPLC

## Selectivity changes



Column: ZORBAX SB-C18 4.6 x 50 mm, 1.8 µm

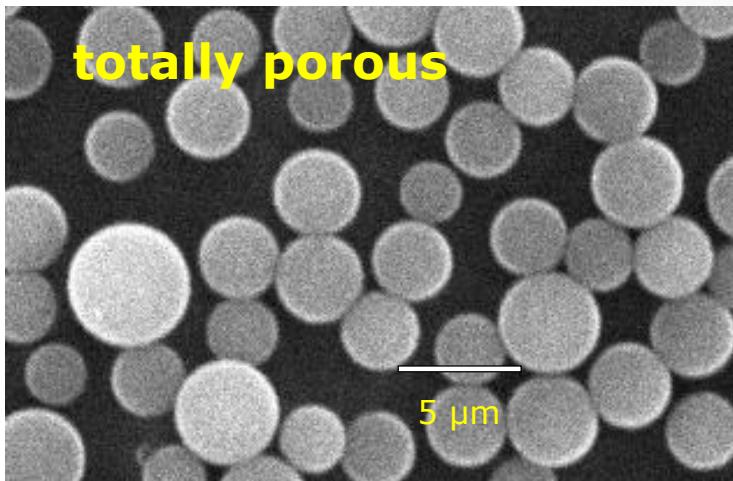
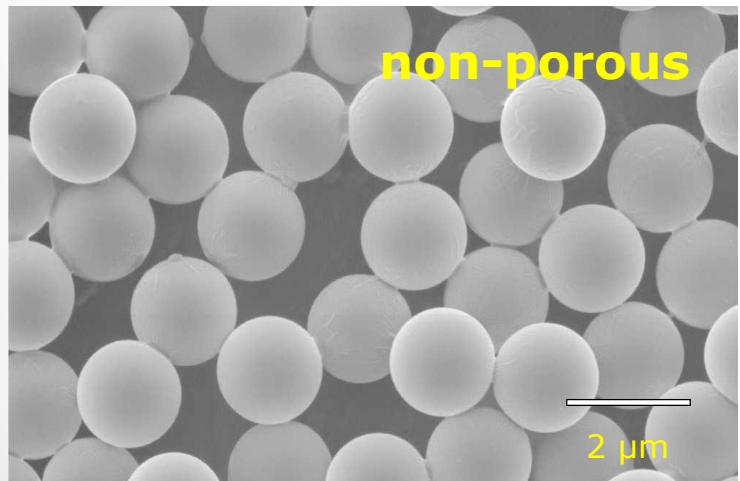
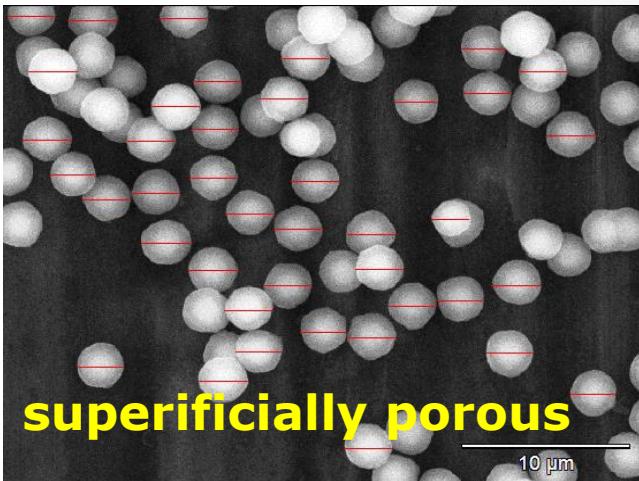
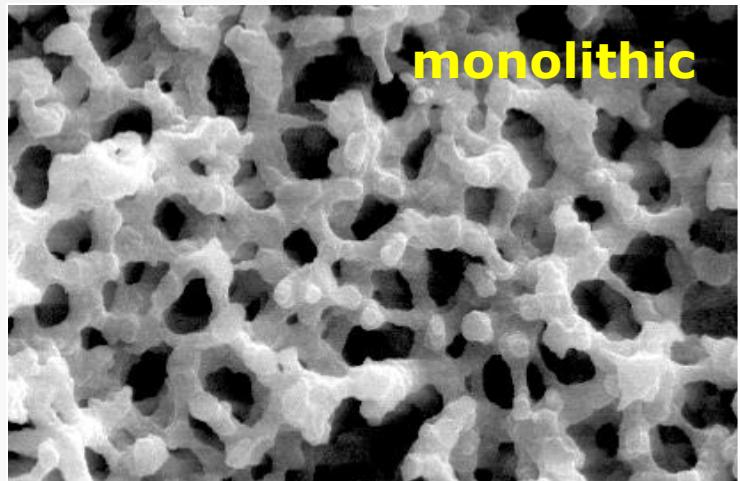
Solvent: A: Water + 0.1% formic acid B: Acetonitrile + 0.1% formic acid (85:15), Flowrate: 1 mL/min

Data and slide courtesy of Dr. Udo Huber, Agilent Technologies, Germany

# Silica-based Column Technology for UHPLC

Particle Morphology	Characteristic Size	Requirements, Applications
<b>Totally Porous (TP) Particles</b>	<b>2.5 – 10 µm</b> <b>8 – 30 nm pores</b>	<b>Standard materials since many years; standard equipment, routine HPLC</b>

# Column Technologies for UHPLC



Photos courtesy of Dr. Bill Barber, Agilent Technologies

# Column Technology for UHPLC

## Superficially Porous Particles

Superficially Porous Particles were introduced already in the 60ties by Cs. Horvath and modernized by J.J. Kirkland et al in the late 90ties (Poroshell 300) and in 2005 (Halo series)

The particle has 2.7  $\mu\text{m}$  outer diameter with a solid core (1.7  $\mu\text{m}$ ) and porous outer layer with a 0.5  $\mu\text{m}$  diffusion path. The average pore diameter is 120 Å. The core has 25% of the particle volume. 75% of the particle volume is porous. (Poroshell 120)

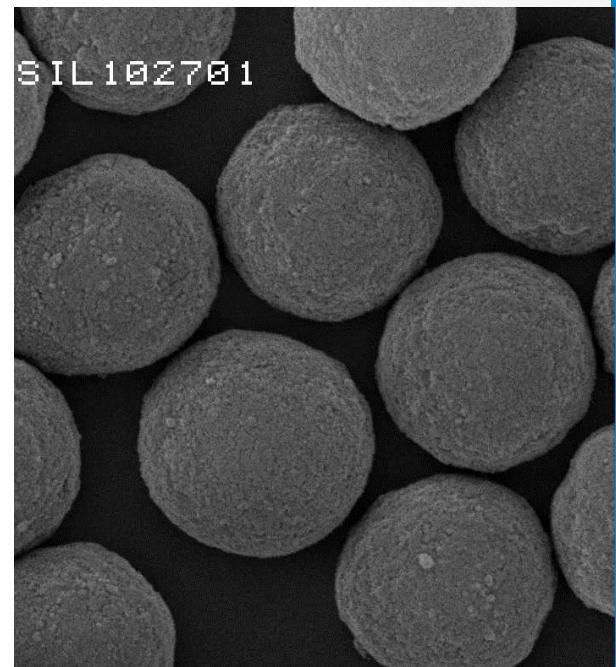
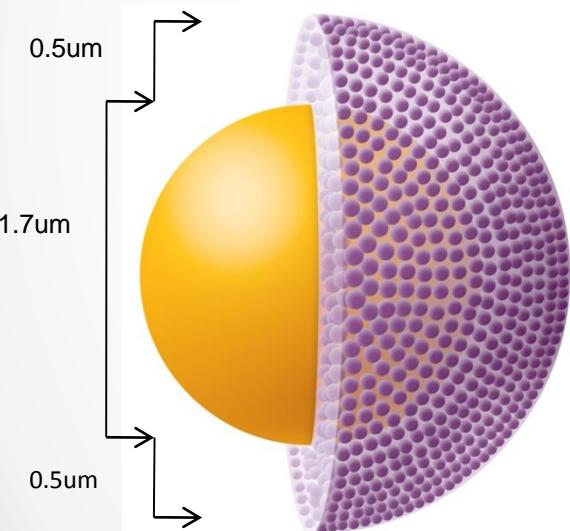
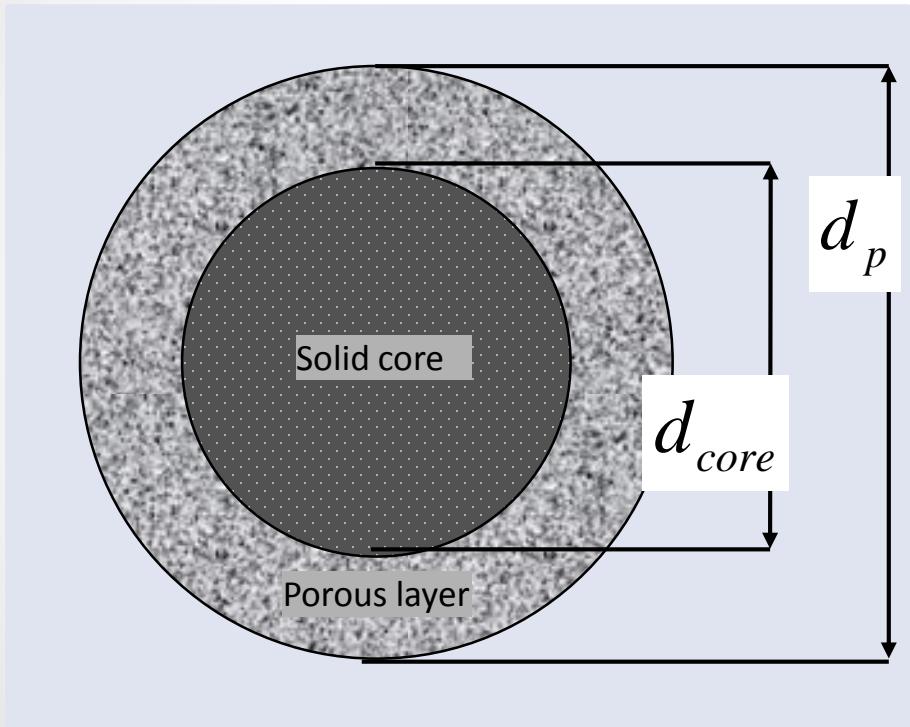


Figure and photo courtesy of Dr. Bill Barber, Agilent Technologies

# Morphology of Superficially Porous Particles



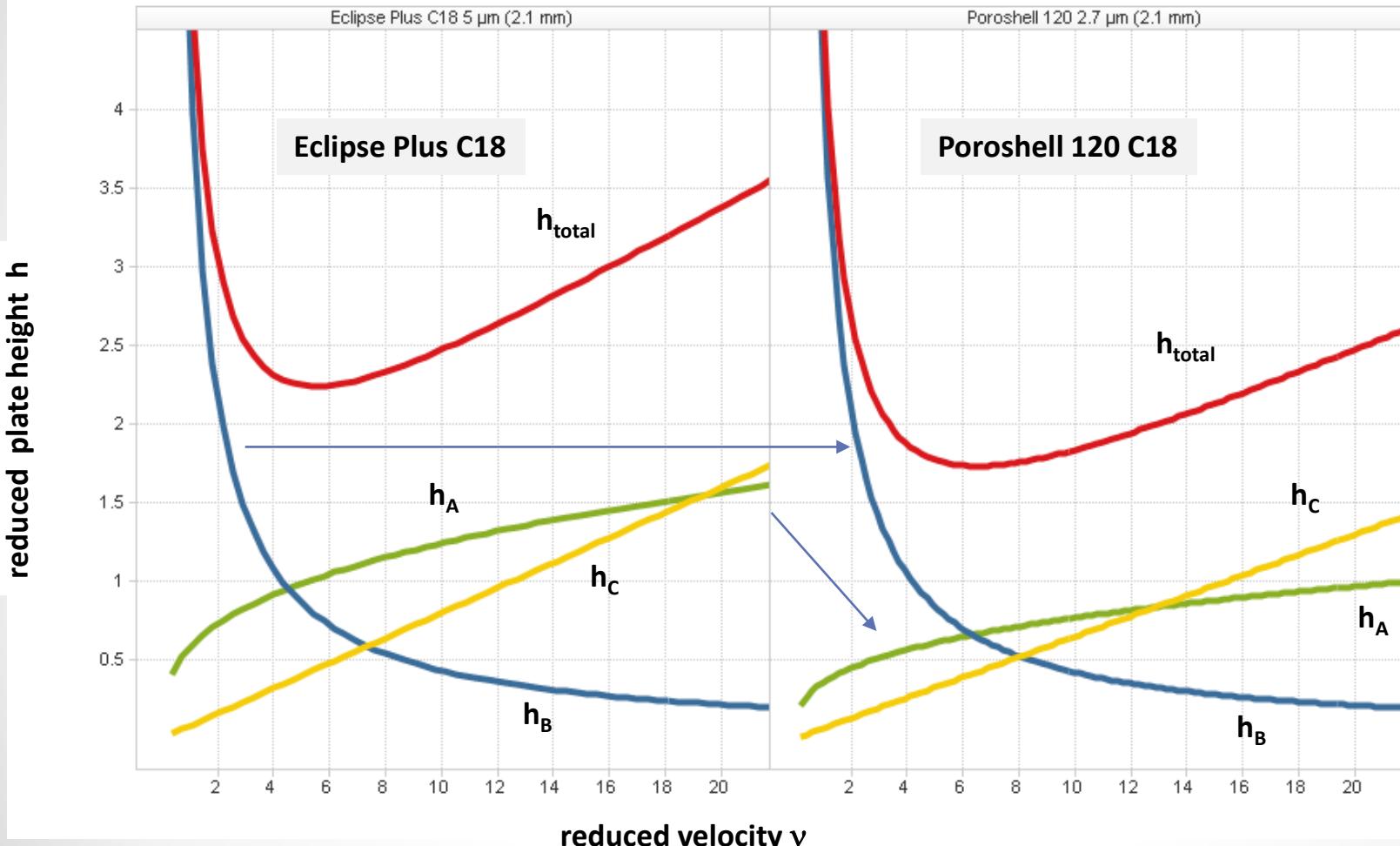
The porous volume fraction  $\varphi$  of a superficially porous particle is given by

$$\varphi_{PV} = 1 - \left( \frac{d_{core}}{d_p} \right)^3 \cong 0.75$$

The internal porosity  $\varepsilon_i$  of a superficially porous particle is assumed to be

$$\varepsilon_i = \varepsilon_{i, \text{fully porous}} \cdot \varphi_{V\text{porous}}$$

# Knox Plot TP and SP Particle Columns



$$h = H / d_p$$

$$\nu = \frac{u \cdot D_m}{d_p}$$

Data and slide courtesy of Dr. Monika Dittmann, Agilent Technologies, Germany

# Summary Column Technology

- Superficially porous particles are an excellent compromise between efficiency of totally porous particles < 2 µm and permeability of larger particles (3 µm).
- The stationary phase volume of a SP particle is about 25% less than a totally porous particle
- The main reasons for the better efficiency of SP particle columns compared with TP particle columns are:
  - Lower contribution to HETP by Eddy dispersion (A-term)
  - B-term contribution is less since there is less volume in the particle
- Silica based monoliths have large thru pores and therefore behave like larger particles. They are more suited for very long columns for high plate number or fast separations with low plate number

# Method Translation

## HPLC → UHPLC Isocratic Separations

- Plate number scales linearly with length and inversely with particle size

$$N \sim \frac{L}{d_p}$$

Same time, better resolution  
Shorter time, same resolution  
Shorter time, lower resolution

Column length (mm)	N, 5 µm	N, 3.5 µm	N, 1.8 µm
150	13050	18650	36250
100	8700	12400	24150
50	4350	6200	12100

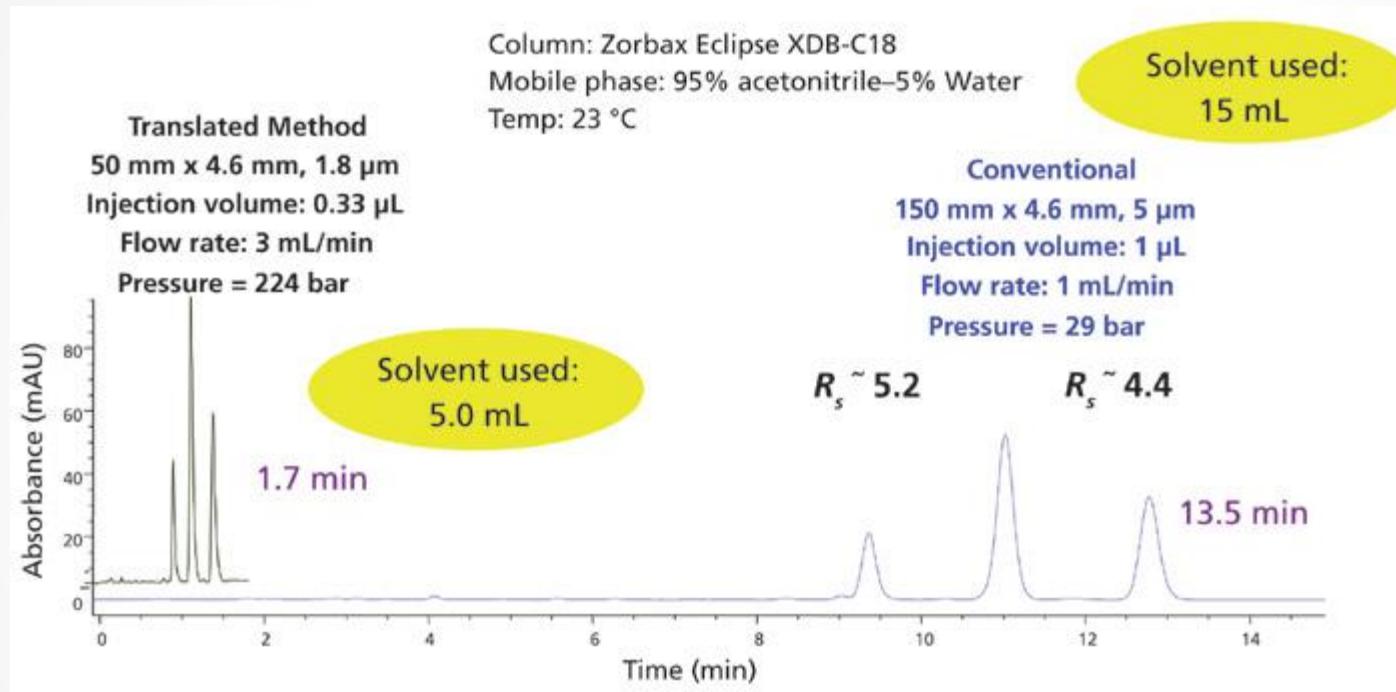
- Flow rate scales quadratic with the column diameter

$$F_2 = \frac{d_{c2}^2}{d_{c1}^2} \cdot F_1 = \frac{2.1^2}{4.6^2} \cdot 1\text{mL/min} = 0.21\text{mL/min}$$

Data and slide courtesy of Dr. Udo Huber, Agilent Technologies, Germany

# Method Translation

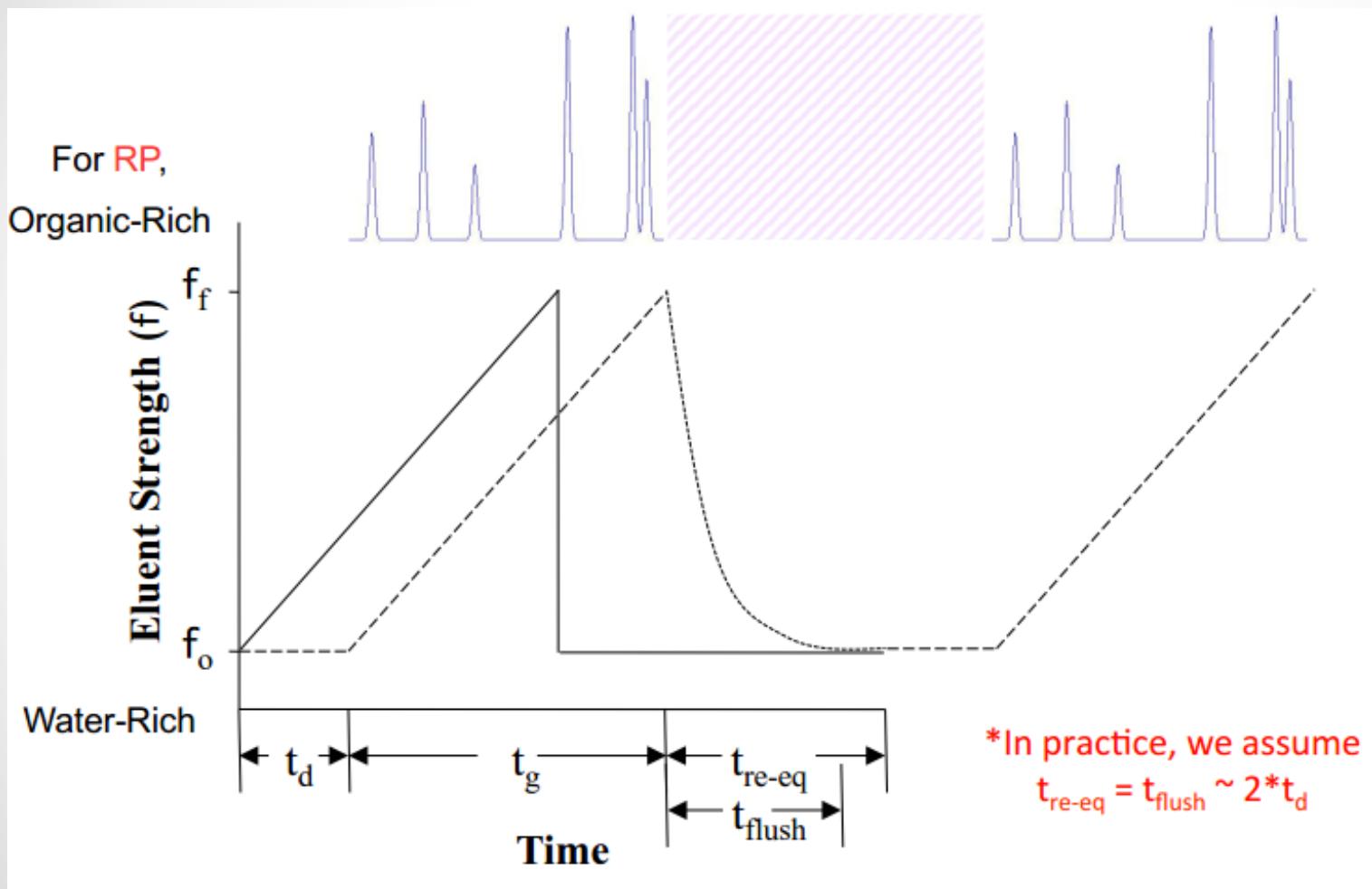
## HPLC → UHPLC Isocratic Separations



Ronald E. Majors, LCGC North America, Volume 29, Issue 6, pp. 476-485

# Method Translation

HPLC → UHPLC Gradient Separations

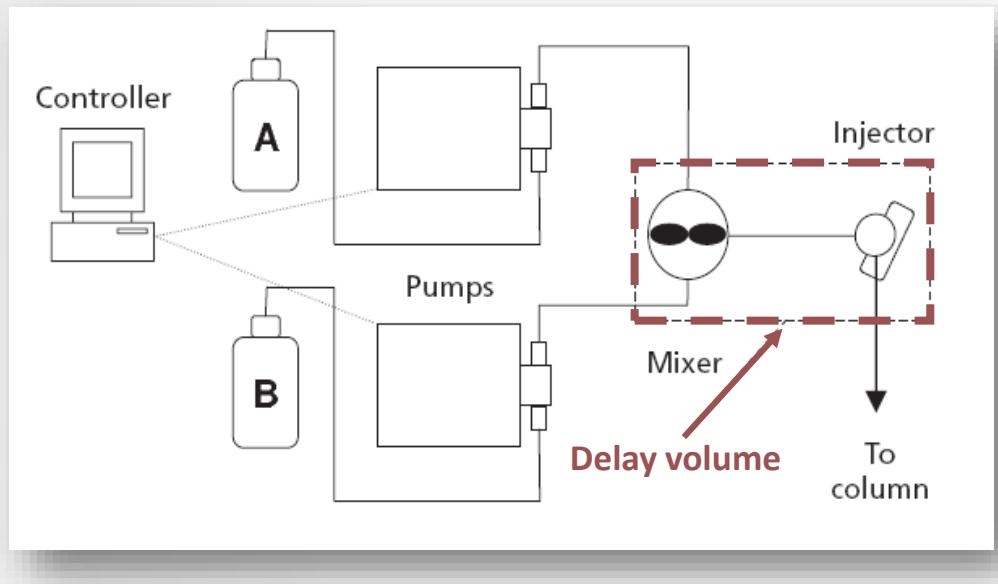


Slide courtesy of Prof. P. Carr & Dr. D. Stoll

# Method Translation

## HPLC → UHPLC Gradient Separations; definition of delay volume

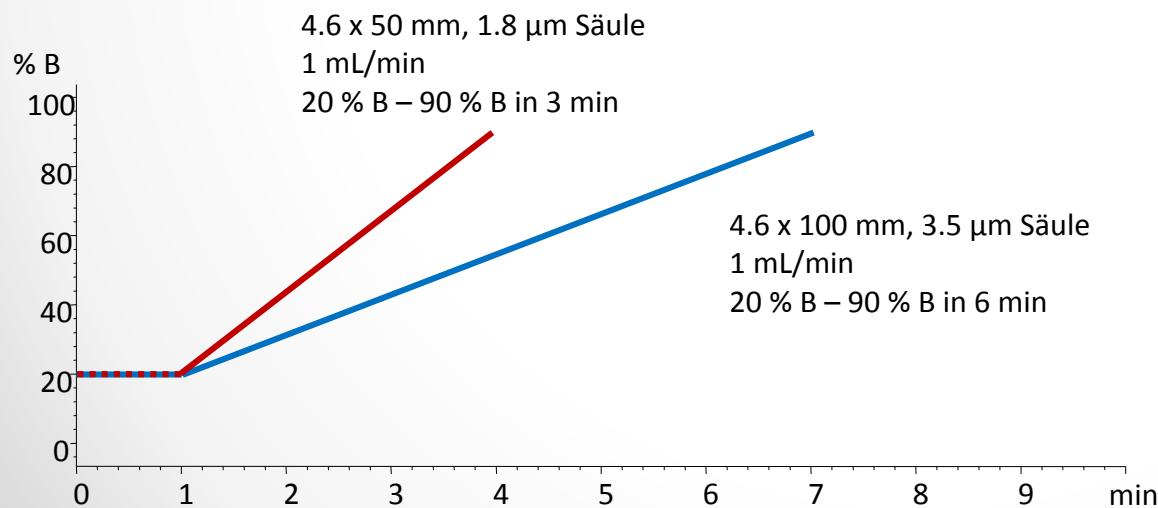
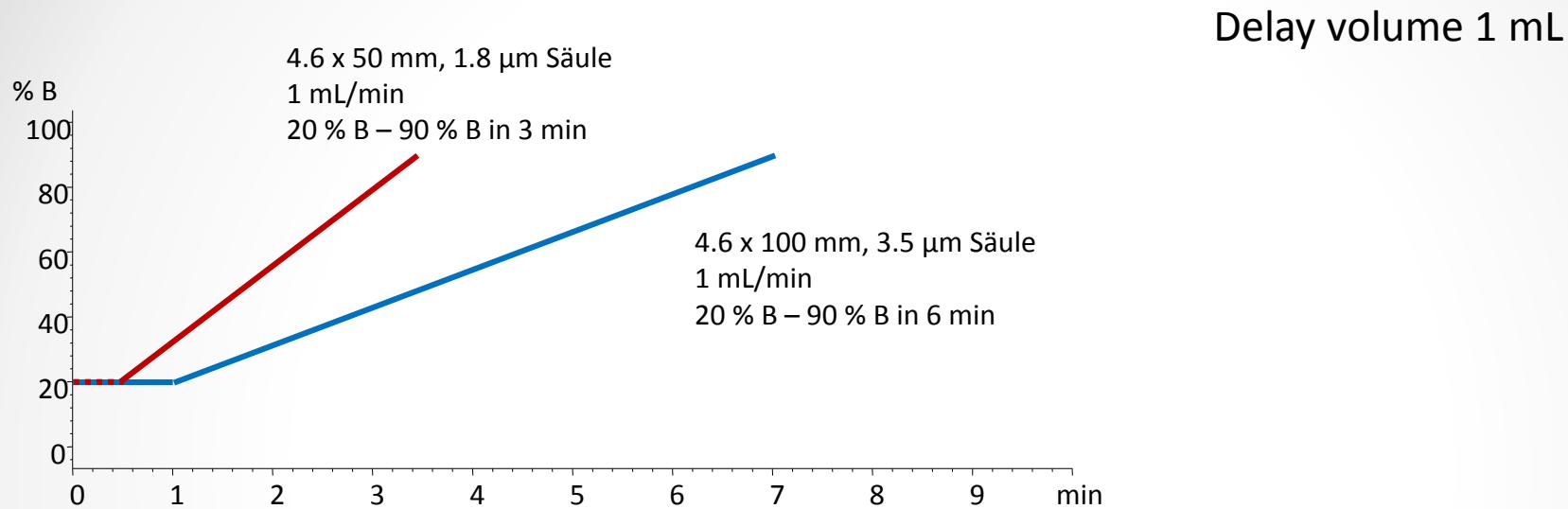
Delay volume or dwell volume defined as the “volume from the point of mobile phase mixing to the column head”



- Delays the arrival of the gradient at the head of the column
- Extends the time by which the solutes on the column are under isocratic separation conditions.

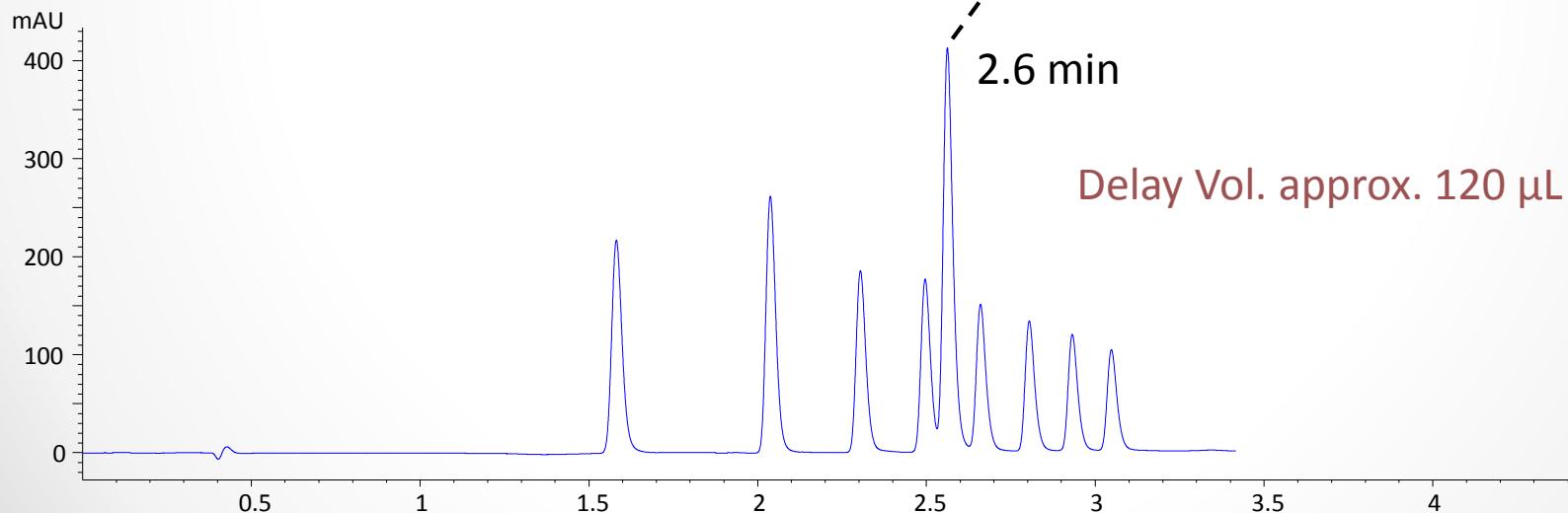
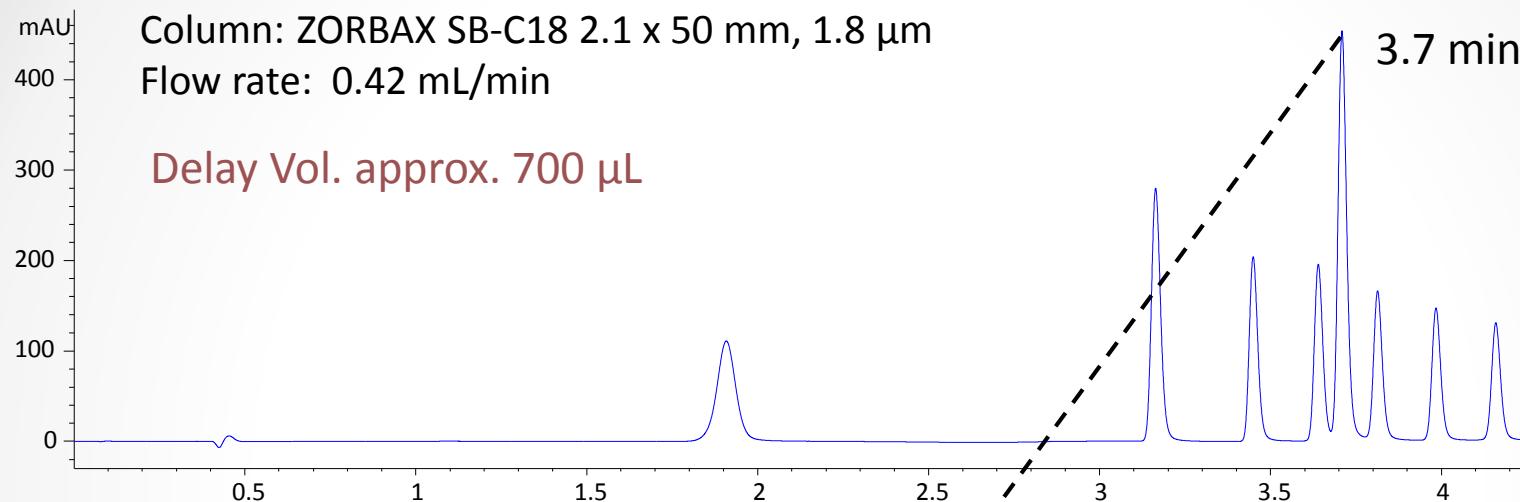
# Method Translation

## HPLC → UHPLC Gradient Separations; Influence of delay volume



# Method Translation

## HPLC → UHPLC Gradient Separations; Influence of delay volume



# Method Translation

## Literature and Software

- <http://www.chem.agilent.com/Library/technicaloverviews/Public/5990-9213EN.pdf>
  - Agilent Technologies LC Calculator App for iPhone (isocratic only)
- <http://www.americanpharmaceuticalreview.com/Featured-Articles/36760-Direct-Method-Scaling-from-UHPLC-to-HPLC-Is-this-feasible-for-Pharmaceutical-Methods/>
- Intelligent System Emulation Technology (ISET) Agilent Technologies for Method Transfer
  - Emulates other HPLC systems (both Agilent and competitors) on Agilent 1290 system
  - Run existing HPLC methods on Agilent 1290 Infinity without modifying your method or system

# Effect of High Pressure on Solvent and Column Properties in UHPLC\*

- Solvent density ( $\rho$ ) resp. specific volume, viscosity ( $\eta$ ), compressibility ( $\chi$ ) and melting point change
- Retention factors ( $k'$ ) and chemical equilibriums
- Solute diffusion coefficient ( $D_m$ ) ↘
- Total porosity of the packed bed ( $\varepsilon_T$ ) ↗
- Column dimensions length and diameter ( $L$  and  $d_c$ )
- Frictional heating causes temperature gradients (longitudinal:  $\Delta T_L$  and radial:  $\Delta T_R$ )

\*M. Martin & G. Guiochon, J. Chrom. A, 1090, 16 , (2005)

# Some Thermal Considerations

- Friction Heating
  - Axial Temperature Gradient
  - Radial Temperature Gradient
- Role of column thermostat method

# Frictional Heating

**Power ( $P$ ) generated in an HPLC column by frictional heat**

$$P = \Delta P \cdot F$$

$$W = J / s = \frac{Nm}{s} = \frac{N}{m^2} \frac{m^3}{s}$$

Equivalent with electrical power  $P=V.I !!!$

# Frictional Heating\*

**Power ( $P$ ) generated in an HPLC column by frictional heat**

$$P = \Delta P \cdot F$$

E.g. Column: 150 mm x 4.6 mm,  $d_p = 3 \mu\text{m}$

$F = 1 \text{ mL/min}$  (mobile phase velocity = 1.4 mm/s, optimal flow rate)

Water ( $\eta = 1.10^{-3} \text{ Pa s}$ )

$K_s = 0.89 \times 10^{-14} \text{ m}^2$

$$\Delta P = 169 \text{ bar} = 1.69 \times 10^7 \text{ Pa}$$

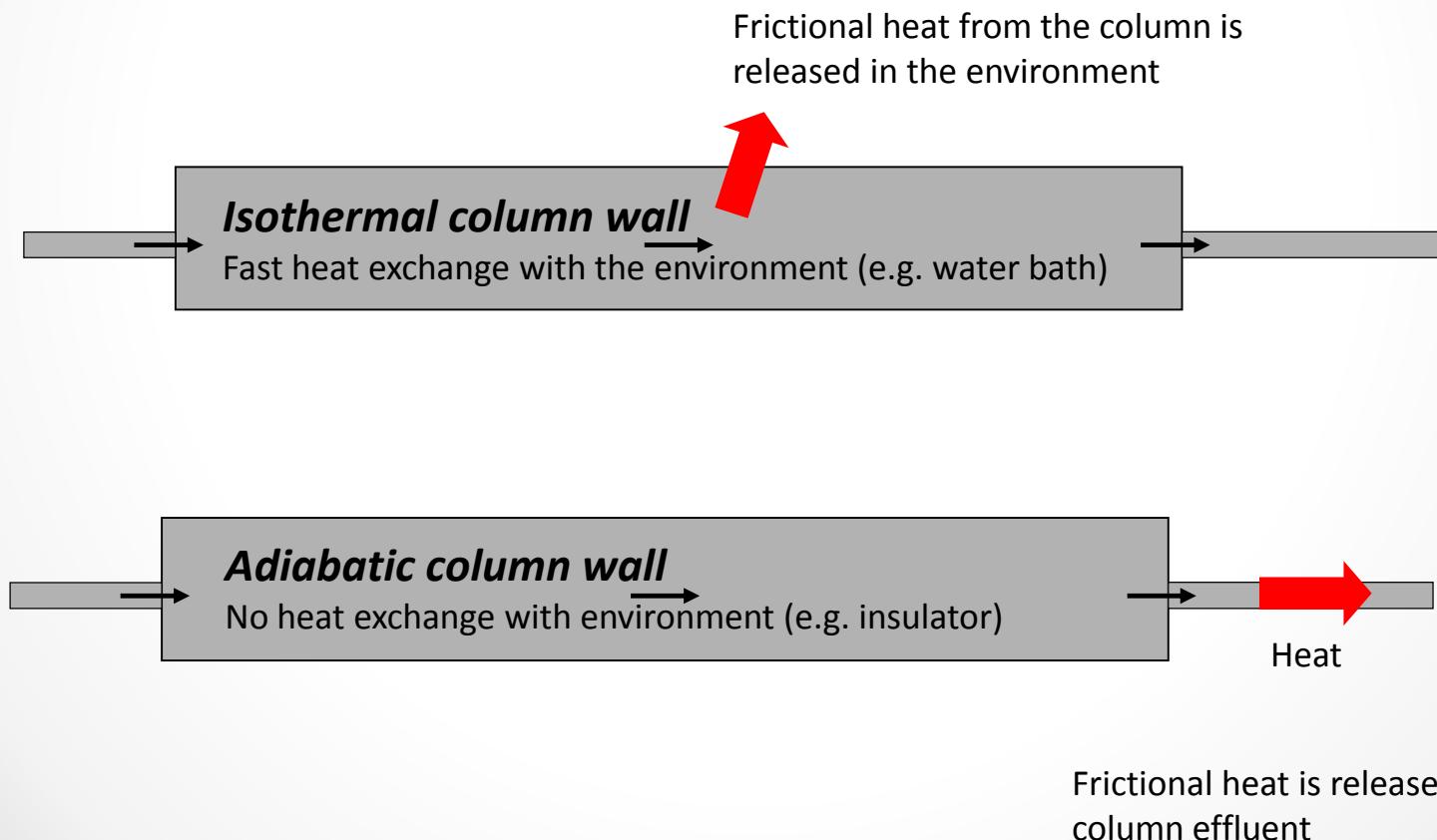
**Power = 280 mW**

\*Colón et al., Analyst, 129 (2004) 503

# Frictional Heating

## Two Limiting Cases for Thermal Environment

Assume Infinitely Thin Column Wall (no thermal mass)



# Importance of Operational Conditions

## Visualisation of solvent velocity

Adiabatic wall = insulated, no heat exchange with environment



Isothermal case = well thermostatted, fast heat exchange with environment



Simulation courtesy of Desmet et al, Free University of Brussels, Department of Chemical Engineering

# Magnitude of $\Delta T_L$ at high inlet pressures\*

$T$ (°C)	$F$ (mL/min)	$\Delta P$ bar	$P$ mW	$\Delta T_L$ (calculated)	$\Delta T_L$ (measured)
25	0.8	670	893	19.3 °C	10 °C
25	1.0	819	1360	23.6 °C	13 °C
40	1.0	749	1250	21.6 °C	13 °C
40	1.1	822	1510	23.7 °C	16 °C

Column: 50 x 2.1 mm; BEH C18; 1.7 µm; ACN/Water – 30/70

Calculated values differ from measured values:

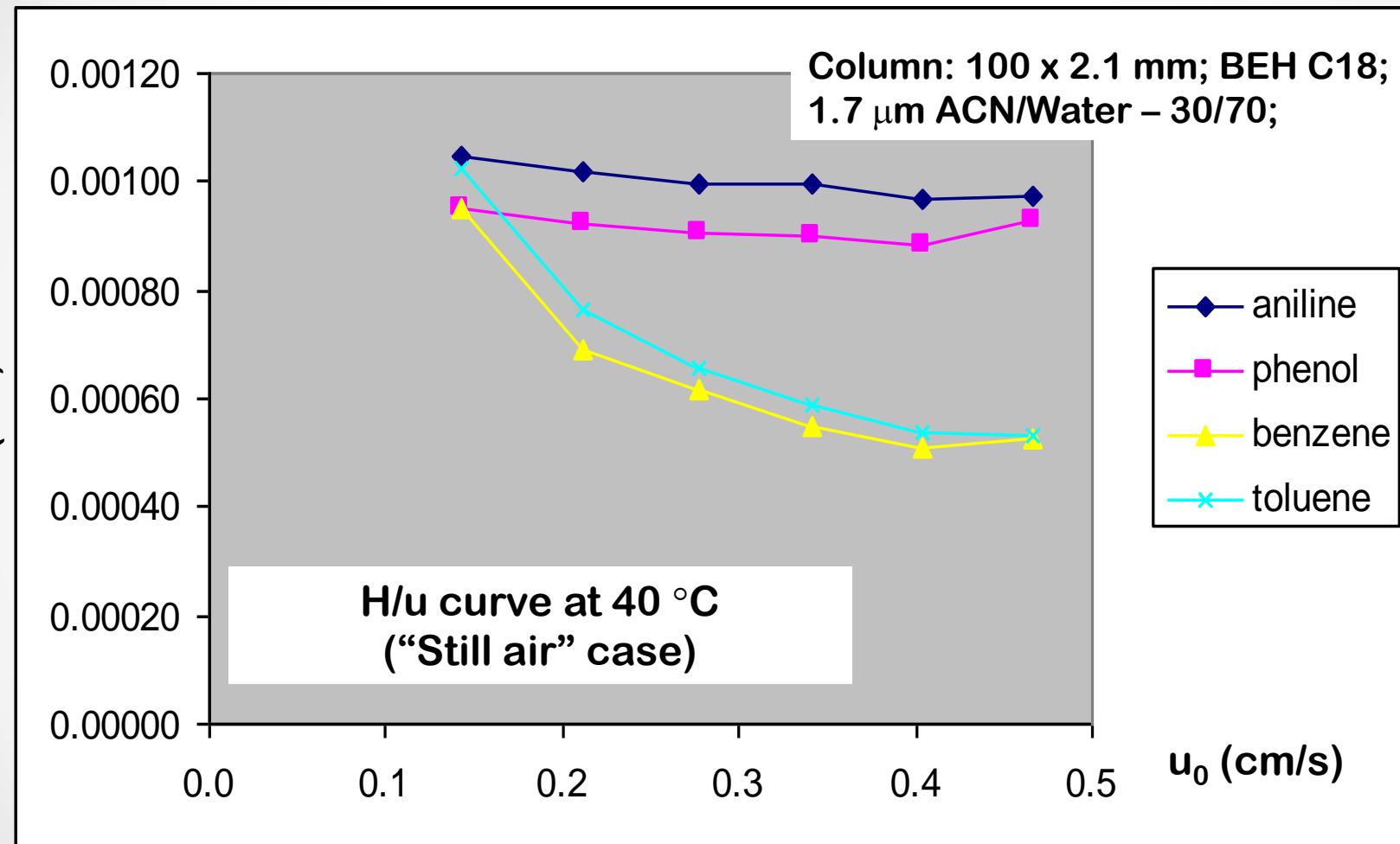
- 1) Heat loss by radiation
- 2) Backflow of heat over column from outlet to inlet

**Longitudinal temperature gradient will not be a problem in practical operation provided that the radial temperature distribution is homogenous!**

\*Sandra et al., J. Chrom. A, 1113, 84 (2006)

# Importance of Operational Conditions

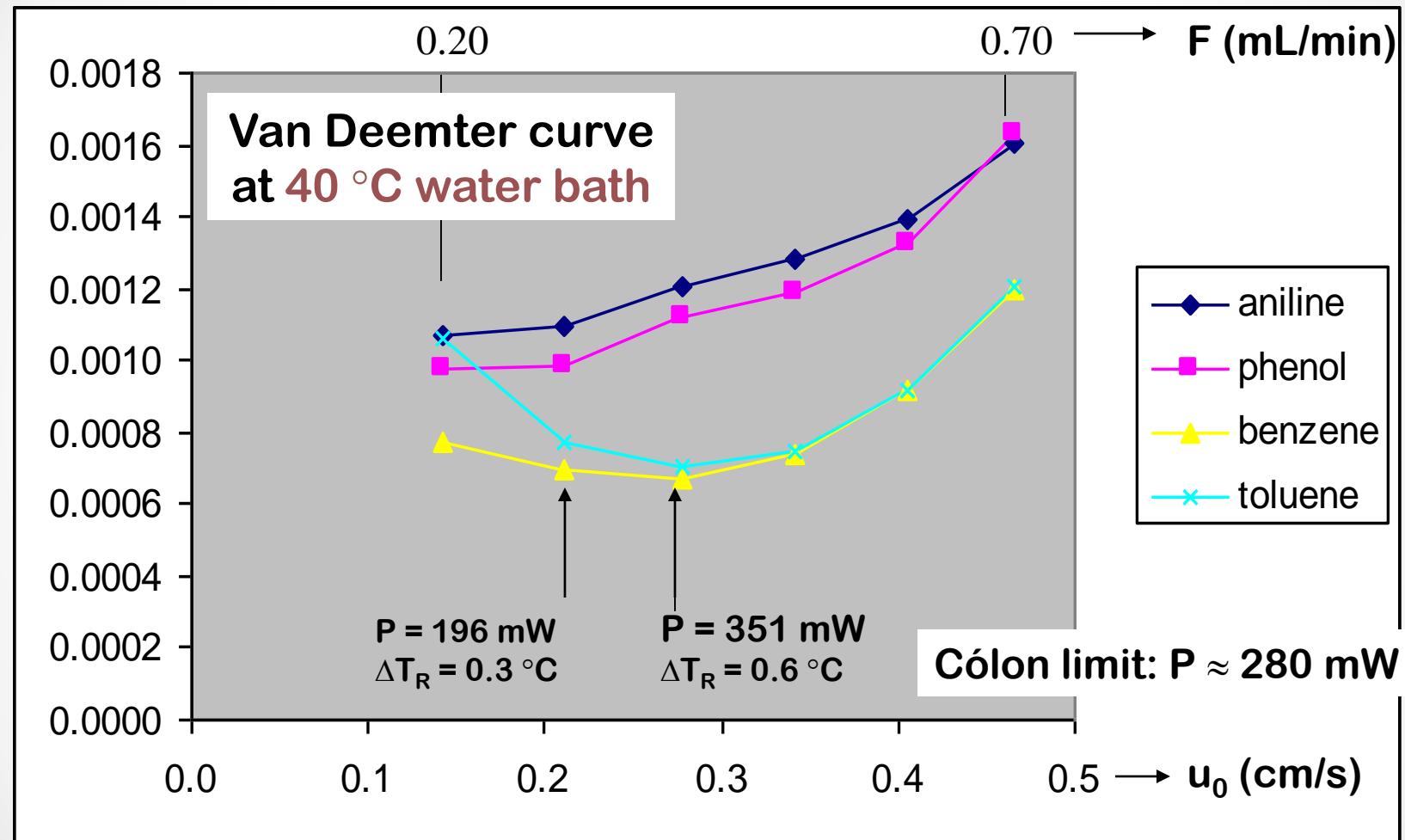
## "Still air" Case\*



\*P.Sandra et al., J. Chrom. A, 1113, 84 (2006)

# Importance of Operational Conditions

## Isothermal Case\*

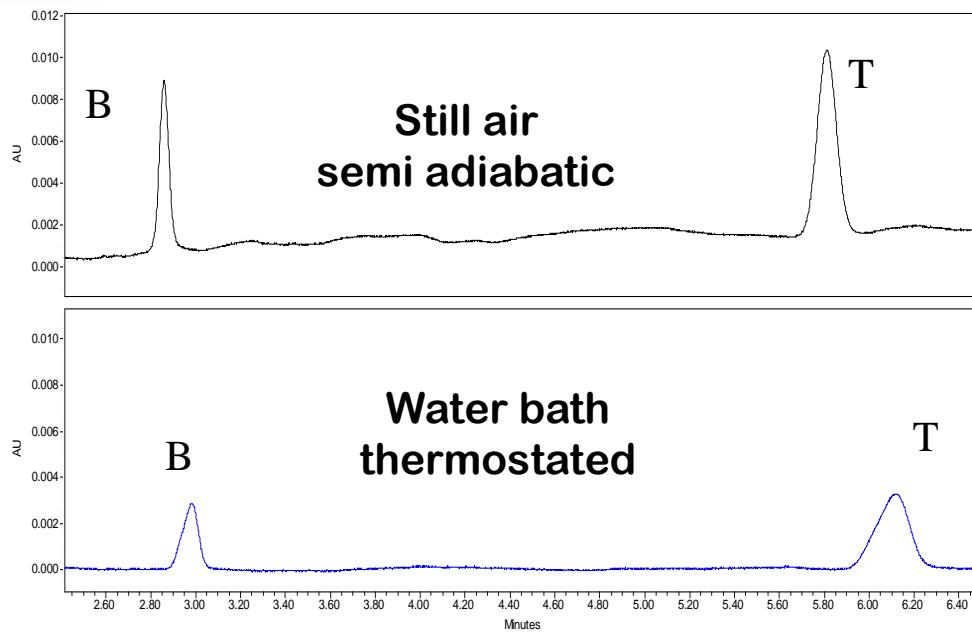


\*P.Sandra et al., J. Chrom. A, 1113, 84 (2006)

# Importance of Operational Conditions

## “Still air” Case vs Isothermal Case\*

Column: 50 x 2.1 mm; BEH C18; 1.7  $\mu$ m; ACN/Water – 30/70, flow rate 0.7 ml/min



$\Delta T_L = 26.1 \text{ }^\circ\text{C}$   
 $N = 20123$

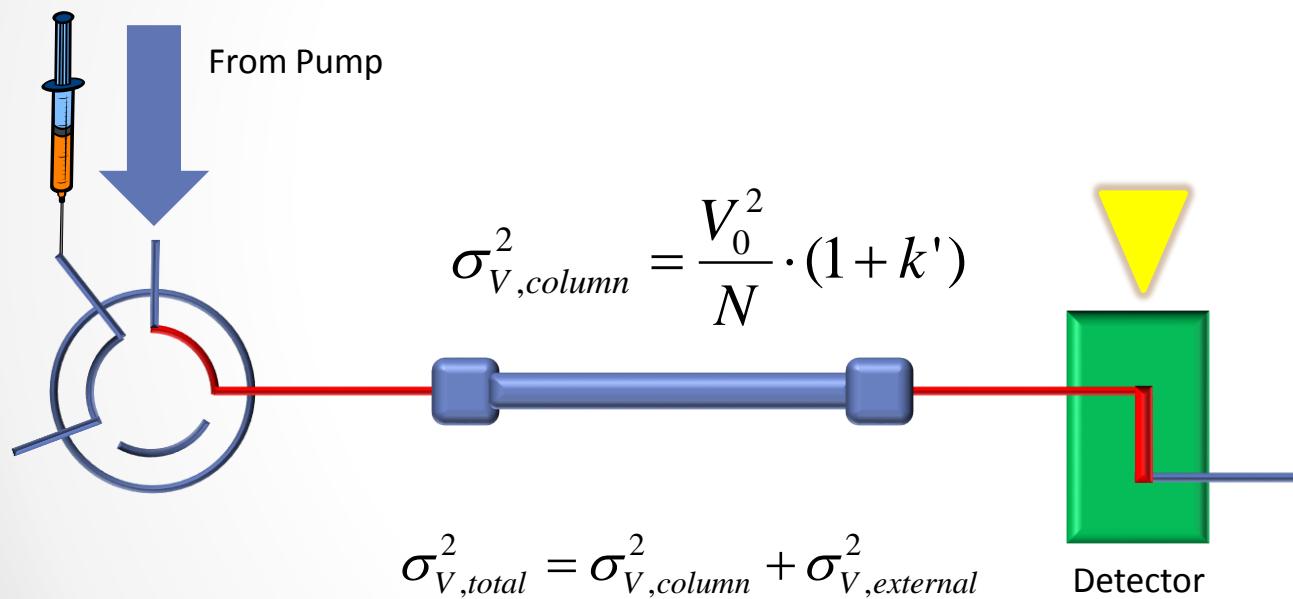
$\Delta T_R = 1.82 \text{ }^\circ\text{C}$   
 $N = 8595$

Marginal influence of  $\Delta T_L$  on peak shape and plate number  
Major influence of  $\Delta T_R$ !

\*P.Sandra et al., J. Chrom. A, 1113, 84 (2006)

# Sources of External Band-Broadening

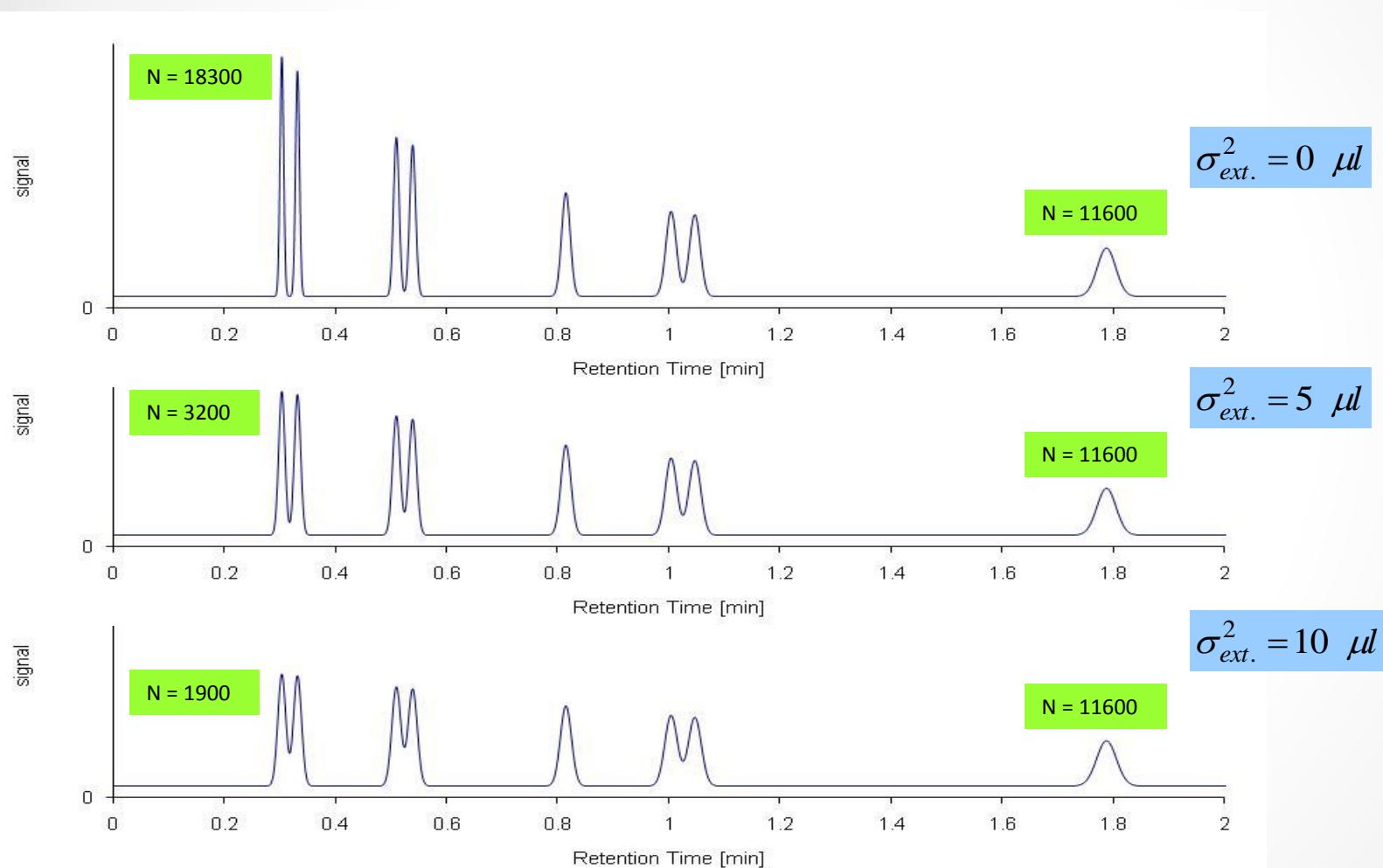
All parts of the system between the point of sample introduction and the point of injection contribute to dispersion.



\*Slide courtesy of Dr. Monika Dittman, Agilent Technologies

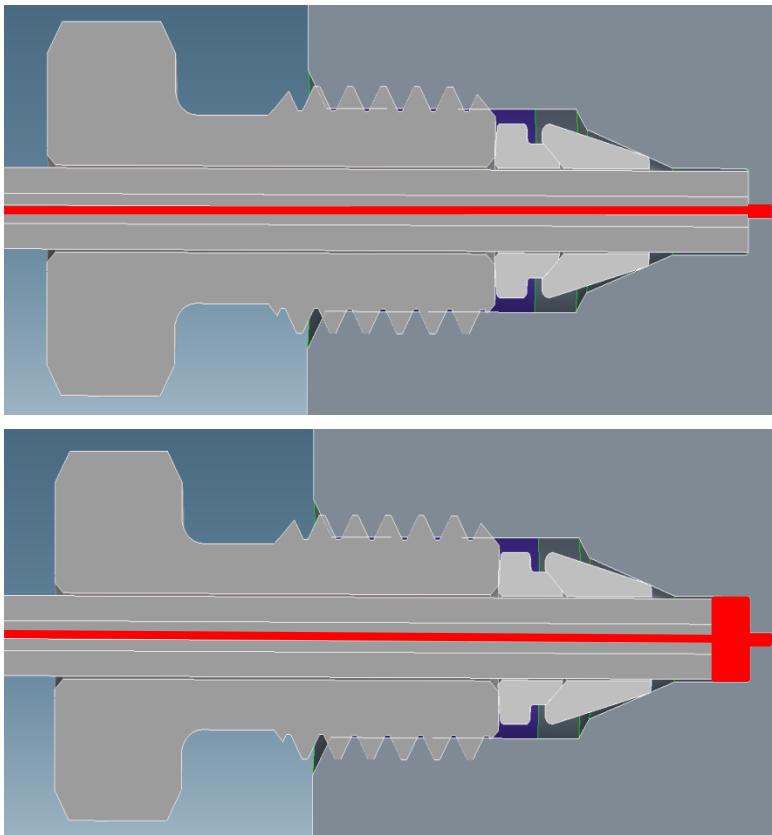
# Effect of External Band-Broadening on N

Effect on 2.1 x 50 mm column with 1.8  $\mu\text{m}$  particles; isocratic elution, calculated example



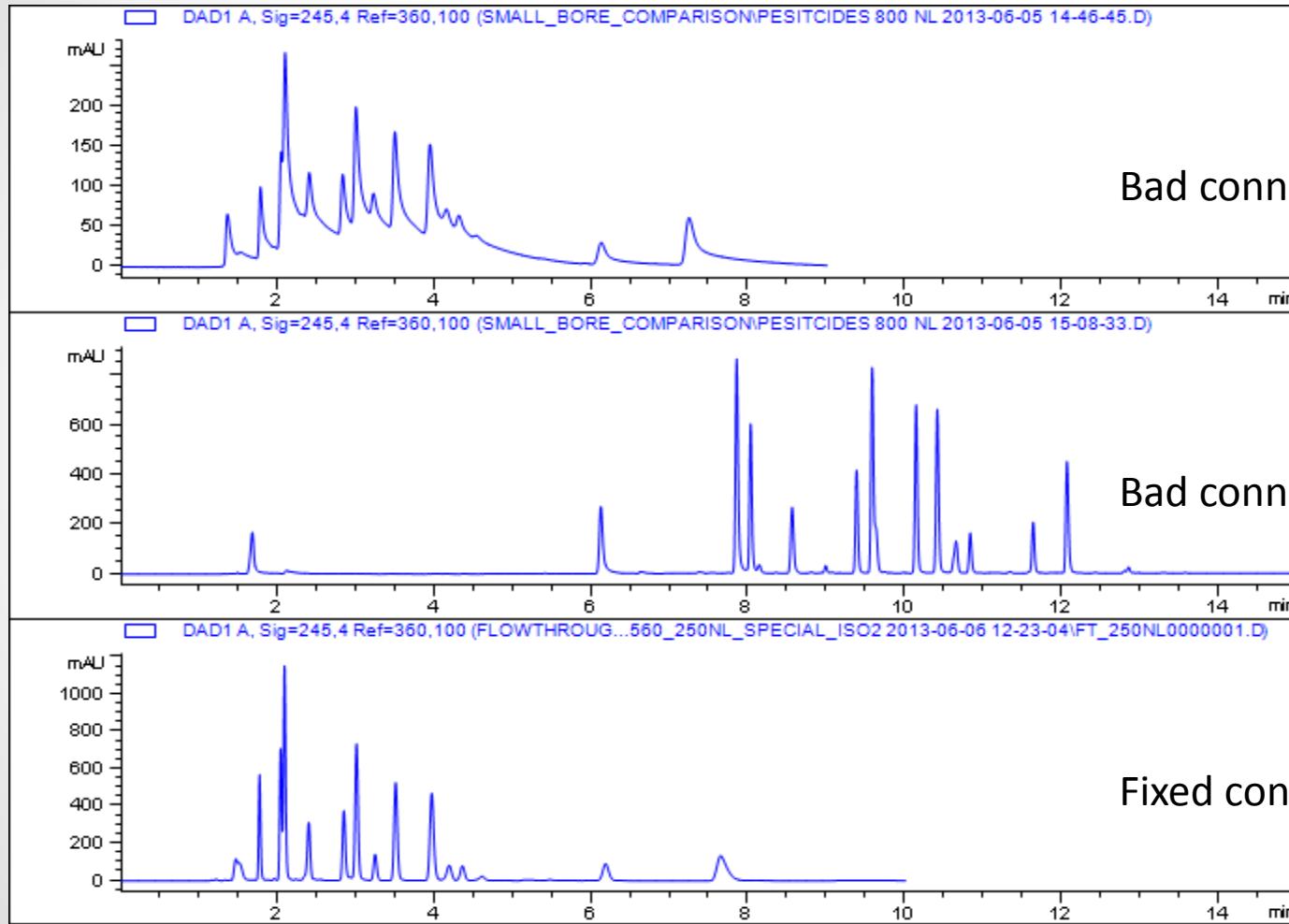
\*Slide courtesy of Dr. Monika Dittman, Agilent Technologies

# Void Volume Caused by Incorrect Fittings



\*Slide courtesy of Dr. Monika Dittman, Agilent Technologies

# Impact of Bad Connection on a 1 mm Column



\*Slide courtesy of Dr. Monika Dittman, Agilent Technologies

# Acknowledgements

- Monika Dittmann, Karsten Kraiczek and other co-workers at Agilent Technologies in Waldbronn, Germany
- Gert Desmet et al., Free University of Brussels, Department of Chemical Engineering

# Thank You for Your Attention

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